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Analysis of cyanobacteria as cosubstrate for anaerobic fermentation of distillery waste

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Declaration of Authorship

I, Aleksandr Marisev, hereby certify that this thesis has been composed by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a Bachelor's degree. This project was conducted by me at the Ostfalia University of Applied Sciences from 05/2018 to 07/2018 towards fulfillment of requirements of Ostfalia University of Applied Sciences and Tampere University of Applied Sciences for the degrees of B. Eng. In Environmental Engineering & Bio and Environmental Engineering under the supervision of Professor Elke Wilharm and Professor Thorsten Ahrens.

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SUMMARY

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In this paper, the biogas and methane potentials were analysed by utilizing cyanobacteria as cosubstrate with distillery waste. Cyanobacteria *Arthrospira platensis* was produced in a tubular airlift photobioreactor and was harvested in weekly basis. As well, the growth rate of *Arthrospira platensis* was observed during each weekly harvest. For better analysis of substrate, batch tests were performed, where one test was distillery waste monosubstrate and the other test was cyanobacteria-distillery waste mix. As well, for comparison, two bioreactors operating in continuous mode were used, one with distillery waste only and the other one with cyanobacteria-distillery waste mix.

It was identified that *Arthrospira platensis* can grow in cases when the standard medium concentration was diluted. Also, the growth rate was identified to be higher by 18 % than the other same culture with the same light. From weekly harvested medium, it was possible to recover 10,62 g/l (in average during total experiment time) of cyanobacteria biomass as the highest amount from one loop. However, it was impossible to reproduce the same growth rate of *Arthrospira platensis* when it was cultivated in digestate medium, by having no cell increase whatsoever, in the end resulting with approximately 25 grams of biomass when harvested at the end of experiment.

From batch test results, biogas production was higher from distillery waste substrate (1111 l/kg VS for distillery waste and 845 l/kg VS) for cyanobacteria-distillery waste mix (20% cyanobacteria and 80% distillery waste proportion)), while the methane production was nearly the same for both substrates, which was 60 % for distillery waste substrate and 61 % for cyanobacteria-distillery waste mix. Hydrogen sulphide concentration was 30 % higher than distillery waste substrate, but at the end of the experiment was not present anymore. During 35 days of continuous fermentation, the results showed the similar biogas production from both substrates (475 l/kg VS for distillery waste and 520 l/kg VS for cyanobacteria-distillery waste mix (same proportion as batch tests), with around 51% average methane concentration). However, by the end of experiment, the reactor with cyanobacteria-distillery waste mix had a higher methane concentration, with a difference of 5-6 %. Although, the hydrogen sulphide concentration was identified to be high from this reactor with an average difference of 30 % (1180 ppm highest).

Key words: *arthrospira platensis,* cyanobacteria, cosubstrate, corn, distillery waste, continuous anaerobic digestion, batch tests, biogas potential, photobioreactor, digestate, growth rate

CONTENTS

ABBREVIATIONS AND TERMS

PBR	Photobioreactor
VS	Volatile solids
DNA	Deoxyribonucleic acid
PUFA	Polyunsaturated Fatty Acids
CO_2	Carbon dioxide
hv	Photon
$C_6H_{12}O_6$	Glucose
CH_4	Methane
N_2	Nitrogen
H_2	Hydrogen
H_2S	Hydrogen sulphide
NH_4	Ammonium
O_2	Oxygen
ppm	parts per million
$\Delta G^{0\prime}$	Gibb's free energy change
CSTR	Continuous-Stirred Tank Reactor
W	Watts
°C	Degrees Celsius
OD	Optical Density
TS	Total solids
FOS/TAC(VFA/TA)	Volatile Fatty Acids divided by Total Acids
NH_4-N	Ammonium-nitrogen
H_2SO_4	Suphuric acid
GC-MS	Gas Chromotography Mass Spectrometry
v/v	Volume to volume ratio
SDP	Silent Discharge Plasma
PTW	Plate-to-Wire

1 INTRODUCTION

From distilleries, 13 liters of distillery waste is produced per every liter of ethanol. Normally, this waste is used as a fertilizer for crops, but sometimes it can also be used for anaerobic digestion [1]. The production of biogas has been already conducted with distillery waste out of several compositions, such as potatoes, maize, grain, molasses and so far, these experiments were successful. It was told, that the biogas plant can operate with distillery waste as the only monosubstrate [2].

Another substrate for anaerobic digestion is *Arthrospira platensis*, also known as *Spirulina*, which belongs to cyanobacteria species. This material is also considered edible, since it is composed 60% out of protein and is not toxic. Nevertheless, there are debates whether this is a suitable substrate for anaerobic digestion. [3] [4]. According to Debowski et al. (2013) [4], there were many anaerobic digestion experiments conducted with cyanobacterial species. The results from anaerobic digestion varied depending on species. Also, this material was used as cosubstrate with energy crops, bringing some improvements to the biogas production.

Microalgae biomass is known for its potential in biotechnology. It is used commercially for example as a high value nutraceutical product (PUFA, pigments, vitamins), nutrition as human supplement, animal supplement, cosmetics and as a tool to clean the water. It has lately been identified that algae can be used as biofuel or can be used to trap CO_2 from the environment (due to high photosynthetic abilities). With these unique features, microalgae biomass is focused on mass production for various biotechnological processes. [5]

Arthrospira platensis is one of the fastest growing and easiest to cultivate cyanobacterium. There are many studies conducted for optimization of *A. platensis* growth rate by utilizing a so called photobioreactor. Normally, in a conical photobioreactor around 510 g /m^3*day (0,51 g/l*day) can be cultivated. The similar amount of biomass was collected by conducting a slightly different experiment, by having a growth rate close to 430 g/m^3*day (0,43 g/l*day) with a tubular photobioreactor that was fed with urea or nitrate as nitrogen source [6]. Also, for open ponds the productivity is somewhat 0,04-0,07 g/l*day [7]. Obviously, there is still much room for improvement towards the growth rate

of such a microorganism, since so many factors can influence the most favorable conditions for optimal growth.

The digested material that comes out from the anaerobic digesters is commonly known as digestate. The consistency of it is partially from microbial biomass and some undigested material. The VS content of such material varies between 2-20%. The important thing about digestate, is that it is rich with nutrients, such as nitrogen, potassium, phosphorus and some trace elements. It is often used as fertilizer, due to high nitrogen content. [8] [9]

2 THEORY

2.1 Microalgae and cyanobacteria

According to phycologists, algae is considered to be any kind of organism that contains chlorophyll *a* (the pigmentation in all the plants that make them look green and helps the plants to photosynthesize [10]), and a thallus which is not developed into roots, stem and leaves. As an exception, cyanobacteria (also called blue-green algae [11]) were also considered into definition of algae, despite of being prokaryotic organism, however, for a long time it was debated whether cyanobacteria are considered as algae species, and, currently, it is acknowledged as a bacterium [12]. Cyanobacteria are described as oxygenic photosynthetic bacteria. [13]

Since cyanobacteria is a group of photosynthetic bacteria with many species. The DNA of these species is not located in the chromosomes, but in the cytoplasm, where the photosynthetic membranes also are, thus having no nucleus. In fact, for all prokaryotic organisms, there are no membrane bounded organelles. [13]

2.1.1 Cellular features of cyanobacteria

Cyanobacteria (and Prochlorophytes) are Gram-negative prokaryotic bacteria which have a cell wall that is composed of three layers. As can be seen from Figure 1, the first is a structural part that consists of murein (also called peptidoglycan layer, as seen in the Figure 1. The next layer is called outer layer (also called lipopolysaccharide layer). Lastly, the cell might have mucilaginous envelope outside of the layers, which are either mucoid sheath, capsule or slime coat. [13]

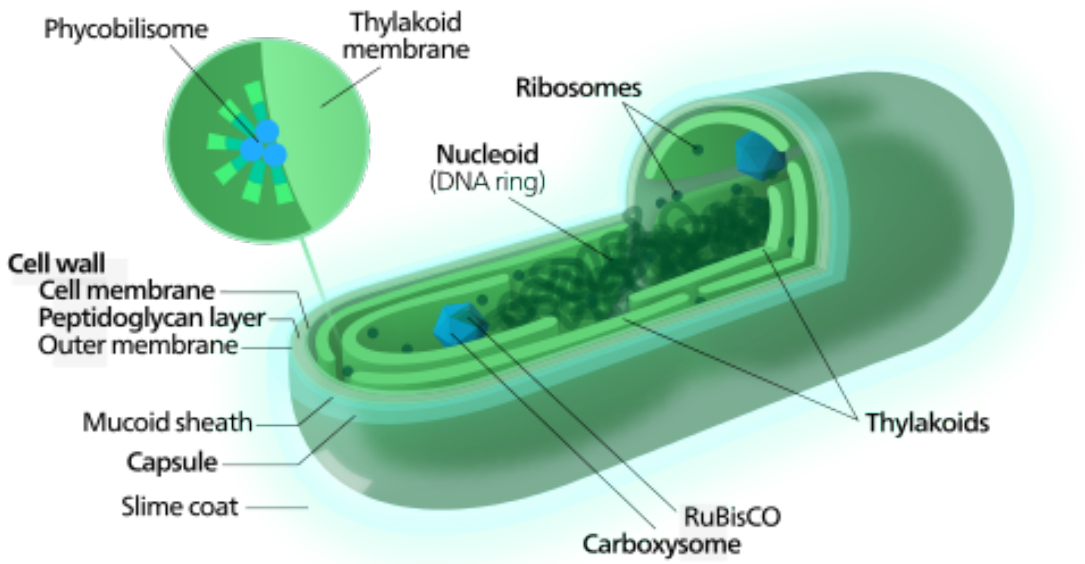


Figure 1.Composition of Cyanobacteria cell [11]

Underneath the cell wall, the cyanobacterial cell has a plasma membrane located, also called plasmalemma which can be 8 nm thick.

The photosynthetic capabilities of Cyanobacteria are located in the thylakoid membrane. Thylakoids are located in the cytoplasm and look like flattened sacs that have phycobilisomes attached to the surface with spacing between each of these phycobilisomes. The arrangement of the thylakoids may vary, these can be in concentric rings, parallel bundles, dispersed etc. [13]

Cyanobacterial cell also contains cell inclusions, where the most common are:

1) Glycogen granules (alpha-1,4- linked glucan) – located between thylakoids and are reserve material
2) Cyanophycin granules (polymer of arginine and aspartic acid) – also located between thylakoids and serve as reserve material
3) Carboxysomes (contains enzyme ribulose 1,5-biphosphate carboxylase-oxygenase) – located in the central cytoplasm
4) Poly-hydroxybutyrate granules (seen as empty holes) – unusual inclusions and a potential source of natural biodegradable thermoplastic polymers, can be absent in some species
5) Lipid droplets (neutral lipid droplets [14]) – located throughout the cytoplasm
6) Gas vacuoles – present in the planktonic forms
7) Ribosomes – distributed throughout the cytoplasm [13]

The typical way the cyanobacteria multiply is by asexual reproduction (although, sometimes transformation or conjugation can be observed). This happens through the binary fission, which can lead to the multiple fissions that can form so called baeocytes (formation of the small cell, which grows into a big one during some period of time [15]). Cyanobacteria also can reproduce by fragmentation (hormogonia). As well, some genera produce akinetes (non-active cell waiting for favourable conditions to grow [16]).

2.1.2 Photosynthesis in cyanobacteria

As described in the previous sub-chapter, the photosynthesis in the cyanobacteria happens with the help of apparatuses called phycobilisomes, which are attached almost everywhere around thylakoid membrane. The photosynthesis process in cyanobacteria is oxygenic. As a short and general description of oxygenic photosynthesis, it is done in the following steps: [17]

$$Pbl + hv \rightarrow Pbl* \quad 1)$$

$$Pbl* Pbl \rightarrow Pbl\ Pbl* \quad 2)$$

$$Pbl* Chl\ a \rightarrow Pbl\ Chl\ a* \quad 3)$$

$$Chl\ a* Chl\ a \rightarrow Chl\ a\ Chl\ a* \quad 4)$$

where,
Pbl – phycobilins
Chl a – Chlorophyll a
hv – photon (Quantum)
* - electronically excited state

As for the chemical reaction of photosynthesis, the reaction can be seen from Formula 5:

$$6H_2O + 6CO_2 \Leftrightarrow C_6H_{12}O_6 + 6O_2 \quad 5)$$

With the help of light, photosynthetic species are able to produce carbohydrates, sugars and other organic compounds (lipids and proteins). Also, with photosynthesis, the photosynthetic oxygenic species can convert CO_2 into oxygen. [18]

2.2 Photobioreactor

Photobioreactor (PBR) is a device which stimulates the growth of phototrophs (microbial, algal, plant cells) by providing necessary conditions for photobiological reaction. The usual design of a photobioreactor is made as a closed system. The reason for this, is that the phototrophic culture in the photobioreactor will not be exposed to contamination, so that the culture will be kept pure. [19]

High variety of organisms are living by the cycle called circadian rhythm. During this, the organisms are differing in their activity during day and night times [20]. As for cyanobacteria, circadian rhythm should be followed in the photobioreactor operation, thus each of these apparatuses in a closed room should have a controlled lighting.

There are many types of photobioreactors existing, therefore the design could be flexible. For example, it can be flat or tubular, horizontal or inclined, vertical or spiral, manifold or serpentine. Each one of these designs have their own pros and cons. The principle of operation can vary as well: for example, there can be air or pump mixed, single-phase reactors, two-phase reactors. Also, the material of photobioreactor can vary, as it can be plastic or glass, rigid or flexible PBR. [19]

2.3 Biogas production

Biogas is a gas which is produced by environments natural processes and by some animals though the process of anaerobic digestion. The anaerobic digestion tract can be met in animals and insects, from example cows, cockroaches, termites etc. Nevertheless, the anaerobic digestion system can also be artificially simulated by men, meaning that it is possible to collect biogas with an apparatus of such purpose. Such devices for production of biogas are often called digesters or bioreactors, and can differ with the design, process, scale. [21]

The biogas is composed of mainly methane (CH_4) and carbon dioxide (CO_2), and can also have trace elements present, such as nitrogen (N_2), hydrogen (H_2), hydrogen sulfide (H_2S), ammonium (NH_4) and oxygen (O_2), which can be seen in details from Table 1 [21]:

Table 1. Common components that make up biogas [21] [22]

	Compound	Composition (% volume of biogas)
Main compounds	Methane (CH_4)	45-70%
	Carbon dioxide (CO_2)	25-50%
Trace elements	Nitrogen (N_2)	<5%
	Hydrogen (H_2)	<1%
	Hydrogen sulfide (H_2S)	50-5000 ppm
	Oxygen (O_2)	<3%
	Water (H_2O)	<10%

2.3.1 Anaerobic fermentation process

The anaerobic fermentation process is commonly known as a process of breaking down the organic matter into smaller monomers by microorganisms, which can be taken up by the same or other microorganisms present in the bioreactor. At the end, the product should be mainly composed out of CH_4 and CO_2. The actual process can be seen from the Figure 2. [22] [21]

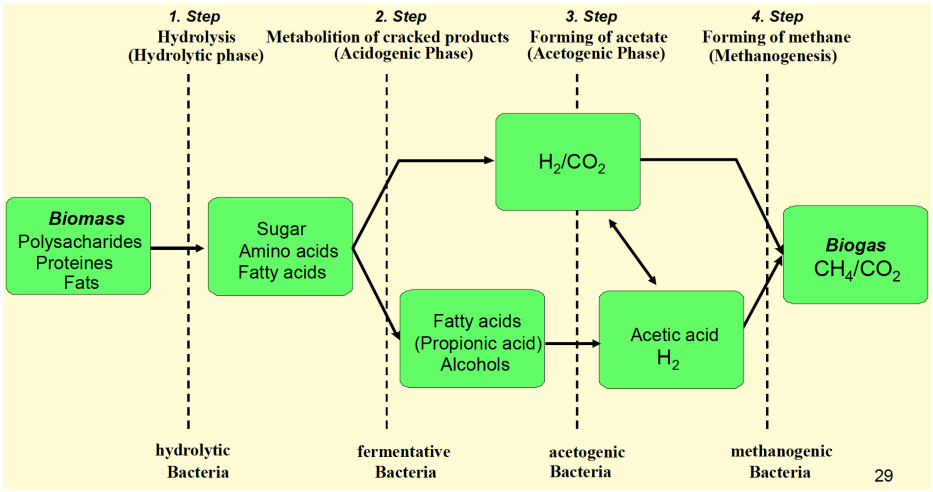


Figure 2. Anaerobic digestion process from start until the end [22]

The figure shows four most important steps in biogas production. Briefly, the process is fastest starting from the left side, and when it goes closer to the right side, which is methanogenic phase, it becomes slower (explained in the next sub-chapters). First, the hydrolytic stage takes place, where the organic matter is broken down into more simple monomers (mainly sugars, amino acids, fatty acids) by hydrolytic bacteria. Afterwards, these simpler monomers are broken down into alcohols and fatty acids during acidogenic phase, by fermentative bacteria (during this stage hydrogen and carbon dioxide are produced as well). Then, acetogenic phase takes place by acetogenic bacteria, where the products from the previous phase used together to form acetic acid. Finally, methanogenesis takes place, where methanogenic bacteria are using up acetic acid and hydrogen/carbon dioxide to form biogas. The composition of the biogas depends on the last two phases, if the amount of hydrogen and produced acetic acid is higher, then the amount of produced methane is also higher, if it is *vice versa*, then the amount of carbon dioxide in the biogas composition will be higher. [22]

The general stoichiometry of biogas production can be seen from Formula 6.

$$C_aH_bO_cN_dS_e + fH_2O \rightarrow gCH_4 + hCO_2 + iNH_3 + jH_2S \quad (6)$$

From Formula 6, a, b, c, d, e can be the numbers, which describe the chemical formula.

More descriptive information about the phases towards formation of biogas and the features of bacteria present during the reactions can be seen from the following sub-chapters.

2.3.1.1. Hydrolysis

Hydrolysis is the first phase towards the production of biogas. During this phase, complex organic matter (proteins, carbohydrates and lipids) is broken down into more simple compounds which are soluble (amino acids, sugars, long-chain fatty acids, glycerine and a minor amount of acetic acid, H_2, CO_2). The occurrence of acetic acid, hydrogen and carbon dioxide happens because the hydrolytic bacteria are excreting enzymes capable of producing such components. The hydrolytic bacteria can be either facultative anaerobes (can live in aerobic and anaerobic conditions) or obligate anaerobes (the one that survive strictly under anaerobic conditions). The time for hydrolytic reaction can vary depending on the type of the material that is introduced to the hydrolytic bacteria. If it is very easy to break down the organic matter, then the phase is faster, if it is very difficult, then *vice versa* (sometimes the process can take days, for example the substrates that contain cellulose, such as solka floc, filter paper, cotton, valonia cellulose, bacterial microcrystalline cellulose [23]). [21]

2.3.1.2. Acidogenesis

During the acidogenesis phase, the produced simpler molecules from the hydrolysis phase (amino acids, sugars, long-chain fatty acids, peptides) are fermented into short-chain fatty acids, CO_2 and H_2. The fermentation happens by facultative and obligate anaerobic bacteria, which are: *Bacteroides, Clostridium, Butyribacterium, Propionibacterium, Pseudomonas* and *Ruminococcus*. The short-chain fatty acids produced, are mainly composed out of acetic, propionic and butyric acids (also, valeric, lactic and succinic acids present in low amounts). As additional by-products of such process, some amount of alcohol can be produced, mainly ethanol. Acidogenesis usually takes minutes to days, and the main products of such process are short-chain fatty acids, that can be used for the next phase. [21]

2.3.1.3. Acetogenesis

The short-chain fatty acids and ethanol from the previous phase are used up by H_2-producing acetogenic bacteria, the reactions go as follows: [21]

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3{}^- + H^+ + 3H_2 \quad \Delta G^{0\prime} = +76{,}1\frac{kJ}{mol} \quad 7)$$

Propionate Acetate

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad \Delta G^{0\prime} = +48{,}1\frac{kJ}{mol} \quad 8)$$

Butyrate Acetate

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2 \quad \Delta G^{0\prime} = +9{,}6\frac{kJ}{mol} \quad 9)$$

Ethanol Acetate

The above reactions depend on the concentration of H_2, if it is too abundant, the aceto-genesis phase would not be maximally productive, as it can be seen from the positive Gibb's energy change ($\Delta G^{0\prime}$) from Formulas 7, 8 and 9. Normally, in an anaerobic system, the excess H_2 is removed by H_2-consuming microorganisms (hydrogenotrophic methano-gens and/or homoacetogens). From this it is possible to state that H_2-producing acetogens and H_2-consuming methanogens/homoacetogens are working together, which is a so called symbiotic (syntrophic) relationship, also called *interspecies H_2 transfer*. [21]

Acetogens are all obligate anaerobes, some examples of species are *Syntrophomonas wolfei* and *Syntrophobacteri wolinii*. The generation time of these bacteria is longer than a week, and the reaction time of acetogenesis phase is very short (products are formed faster). [21]

2.3.1.4. Homoacetogenesis

Homoacetogenesis is the phase where acetate is also produced, but in a different way than during acetogenesis phase. There are two types of homoacetogenic bacteria which are involved in these reactions – autotrophs and heterotrophs. Homoacetogenic autotrophs take up CO_2 and H_2 to produce acetate, where CO_2 is a carbon source and H_2 is an electron donor, as it can be seen from Formula 10. Homoacetogenic heterotrophs take up organic compounds (i.e. formate and methanol) as carbon source to produce acetate as well. [21]

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O \qquad \Delta G^{0\prime} = -104{,}6\frac{kJ}{mol} \quad 10)$$

CO_2 source Acetate

Afterwards, the produced acetate (methyl and carboxyl groups) is oxidized to CO_2, by producing H_2. The oxidizing bacteria are called *acetate-oxidizing bacteria* and they work together as syntrophic association with hydrogenothrops (hydrogenotrophic methanogenesis sub-chapter). The chemical reaction can be seen from Formula 11.

$$CH_3COO^- + 4H_2O \rightarrow 4H_2 + 2HCO_3^- + H^+ \qquad \Delta G^{0\prime} = +104{,}6\frac{kJ}{mol} \quad 11)$$

Acetate

2.3.1.5. Methanogenesis

Finally, the reaction where the methane is produced is carried out by the microorganisms, that are classified as *Archaea*. These microorganisms are strictly anaerobic and are able to produce CH_4 through aceticlastic and hydrogenotrophic pathways. The growth rate of such microorganisms is very slow, and they are very sensitive to the environmental conditions, such as pH, temperature, inhibitory compounds etc. [21]

Acetotrophic/Aceticlastic methanogenesis is the process where acetate is metabolized directly to CH_4. In details, the methyl group of acetate is reduced to CH_4, by following series of chemical reactions and the carboxyl group is oxidized to CO_2. There are two genera of methanogens, which are *Methanosaeta* and *Methanosarcina*. *Methanosaeta* are aceticlastic methanogens which take up only acetate. The generation time of *Methanosaeta* is 1-2 days when provided acetate. *Methanosarcina* are the ones that can be both acetotrophic and aceticlasic, of which generation time is 3-9 days and that are also able to grow with low acetate levels. The reaction of methane production during this phase can be seen from Formula 12. [21]

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad \Delta G^{0\prime} = -31\frac{kJ}{mol} \quad 12)$$

Acetate

As for hydrogenotrophic methanogenesis, hydrogenotrophs reduce CO_2 to CH_4. In order to do that, these microorganisms are using the produced H_2 and CO_2 from the previous

chemical reactions (Formulas 7, 8, 9 and 11). The hydrogenotrophic microorganisms that act in this phase are: *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanopyrales, Methanosarcinales* and *Methanocellales.* Also, several hydrogenotrophs are able to use formate as their electron donor, as can be seen from Formula 13. [21]

$$4HCOO^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^- \qquad \Delta G^{0\prime} = -130{,}4\frac{kJ}{mol} \qquad 13)$$

Formate

2.4 Continuous anaerobic fermentation

Continuous anaerobic fermentation (also called digestion) is a system where the substrate is added in a daily manner, while some part is taken out from the fermenter. The advantages of such fermentation are that it has a shorter processing time, by utilizing the same holding capacity as for example batch fermentation. As well, the product is more stable during this fermentation (quality can be the same, if for example yeast is produced). Also, is much easier to have instruments adjusted for continuous fermentation system, which can save money, since the equipment that is used can be the same for different processes. [24]

Such a fermenter can also be called CSTR (Continuous-Stirred Tank Reactor), which means that it has a stirring option in the reactor. The stirring can happen for the entire time or by intervals. It is simple to build such reactor and it is easy to operate, the usual time for operation of such system is between 20-50 days. One huge advantage over any other fermentation strategies, is that the substrate gets diluted quickly, providing a less toxic environment for the microorganisms (concentration of possible toxic substances decreases). [21]

2.5 Batch fermentation

Batch fermentation is a very simple option of anaerobic digestion. The idea is that the fermenter is filled up with the substrate and microbes. After that, the fermenter is left for a required period of time with required temperature set to digest the material (while being daily mixed), and in the end the products are collected [24] [25]. Such an experiment can

show the potential of a substrate to produce biogas/methane in a smaller scale. With the data collected, it is possible to see whether the substrate is being digested to its highest potential when used in a larger scale. When batch system is operated, the quality of the product differs by each batch. This kind of fermentation strategy is often used for yeast production, beer brewing [22].

3 MATERIALS AND METHODS

The main task was divided into three parts, first part was to collect the cyanobacteria biomass and the second part was to set up bioreactors to run continuously by feeding 5 times a week. The third part was to run batch tests for 35 days.

For the work, *Arthrospira platensis* cyanobacterium was used; it was cultivated in the tubular photobioreactor and then used as cosubstrate for anaerobic digestion. More details can be seen from the sections below.

3.1 Preparation of standard medium for *A. platensis*

For the cultivation of Arthrospira platensis, standard medium was prepared according to the Table 1.

Table 1. Standard medium for Spirulina calculated for 1 liter of solution

Standard medium for *Spirulina* (1 liter)			
Solution I:	**To add**	**Solution II:**	**To add**
$NaHCO_3$	13,61 g	$NaNO_3$	2,50 g
$NaCO_3$	4,03 g	K_2SO_4	1,00 g
K_2HPO_4	0,50 g	NaCl	1,00 g
Distilled water	500,0 ml	$MgSO_4 * 7H_2O$	0,20 g
		$CaCl_2 * 2H_2O$	0,04 g
		$FeSO_4 * 7H_2O$	0,01 g
		EDTA (Titriplex III)	0,08 g
		Micronutrient Solution	5,0 ml
		Distilled water	500,0 ml

As a task for the standard medium preparation, the necessary amount of each chemical was added. First, the solution I was prepared and mixed with a stirring magnet. Then, the solution II was prepared in the sample container, while the solution was stirred (in order to ensure proper dissolution of the chemicals). Before, the preparation of standard medium solution, the micronutrient solution was prepared according to the recipe in Table 2.

Table 2. Micronutrient solution

Micronutrient solution	To add
$ZnSO_4 * 7H_2O$	1 g
$MnSO_4 * 4H_2O$	1 g
H_3BO_3	2 g
$Co(NO_3)_2 * 6H_2O$	0,2 g
$Na_2MoO_4 * 2H_2O$	0,2 g
$CuSO_4 * 5H_2O$	0,005 g
Distilled water	981 ml
$FeSO_4 * 7H_2O$	0,7 g
EDTA (Titriplex III)	0,7 g

As it can be seen from Table 1, the micronutrient solution is also a part of the recipe. Therefore, to complete with the standard solution, needed amount of micronutrient solution was pipetted to the standard solution, after which the standard solution was ready to be transferred to the loop.

3.1.1 Operating photobioreactor

As a preparation part of this experiment, it was important to be introduced to an apparatus called photobioreactor (see Picture 1). This device is separated into 6 different loops made of plastic (two with 10 liter volume and four with 20 liter volume), where each loop can have a different culture of algae growing inside with a prepared culture medium. The principle of operation is that there is a light source located on top side and vertical side beside the loop. The loops had different light intensities, which can be seen from Table 3. The contents of the loop (cyanobacteria in the culture medium) are circulated with air flow inside (not higher than 2-3 l/min air circulation), giving an effect of natural water flow, the heating of the loops is also available, where the loops can be heated up to 24-25 °C, if the room temperature was too low. The idea of the light source is that it maintains the growth of cyanobacteria by providing it necessary conditions to photosynthesise, from which cyanobacteria are releasing O_2. In addition, in order for cyanobacteria not to stick to the surface of the loops, plastic pieces were as well circulated in the culture medium with cyanobacteria to avoid this happening.

Picture 1. Photobioreactor in a harvesting mode, where loops 6 (liquid digestate), 4, 3 and 2 are used (Arthrospira platensis) for thesis.

Table 3. Light intensities of the loops

Loops	Light intensity top lighting (W/m^2)	Light intensity vertical lighting (W/m^2)
Loop 2	38	16
Loop 3	36	17
Loop 4	31	15
Loop 6 (digestate)	17	20

3.1.2 Measurement of optical density

The loops that were used for experiments were tested for their OD in a weekly basis. For this, the samples of each loop were taken and brought to spectrophotometer configured to 750 nm wavelength. First, the blank value was used to configure the instrument. Afterwards, sample was run three times (measuring cuvette was refilled each time with the new sample and measured). Each of the loops was tested this way, and the average of

three measurements was taken. By following the average value, it was possible to identify how much cyanobacteria was needed to be harvested. The data that was recorded, can be seen from Appendices 1, 2 and was the base for making graphs that can be seen in "Results" chapter.

3.1.3 Harvesting of *A. platensis* biomass from loops

The aim of operating photobioreactors, was to collect the biomass of *Arthrospira platensis*. In order to do that, the loops that were used for biomass collection were harvested and newly inoculated/diluted with fresh medium/filtrate to get weekly harvest. In simple terms, they were diluted, and in order to identify what dilution factor was required to be, OD of the loops was measured.

For the collection of biomass, a tube system and underpressure was used. The device was composed out of three hoses, a flow regulator and a syringe, as it can be seen from Picture 2.

Picture 2. Extraction of loop contents for algae biomass harvest

Also, from Picture 2 it can be seen that a plastic beaker with a capacity of 5 litres was used as a container for the extracted contents of the loop (in this case *A. platensis* in the culture medium). With this beaker, it was possible to see how many litres were taken from the loop, so that it can be known how much medium solution should be prepared as a replacement of the taken amount.

When the beaker was filled with the required 5 litres of loop contents, the beaker was then brought to the prepared empty beaker with the filter (mesh size 63 μm) placed on top of it, as it can be seen from Picture 3.

Picture 3. Filtered contents of the loop (on the filter algae biomass and in the beaker filtrate)

The cyanobacterial cells were collected from the filter with a spatula, transferred to the plastic bag and weighed for the record of total collected fresh biomass, afterwards stored at -18°C.

3.2 Preparation of batch tests

Before preparing the batch tests, the right amounts of substrate/inoculate were calculated in the following formulas [26] [27] [28]:

$$m_{substrate}[g] = \frac{0{,}5 * VS_{inoculate}[\%] * 1500g}{VS_{substrate}[\%] + (0{,}5 * VS_{inoculate}[\%])} \quad 13)$$

where,

$m_{substrate}$ – fresh mass of the substrate [g]
$VS_{inoculate}$ – volatile solids of inoculate (sludge) [%]
$VS_{substrate}$ – volatile solids of substrate [%]

$$m_{inoculate}[g] = 1500g - m_{substrate}[g] \quad 14)$$

where,

$m_{substrate}$ – fresh mass of the substrate [g]
$m_{inoculate}$ – fresh mass of the inoculate (sludge) [g]

$$\frac{m_{VS,inoculate}[g]}{m_{VS,substrate}[g]} \leq 0{,}5 \quad 15)$$

where,

$m_{VS,\ inoculate}$ – volatile solids mass of inoculate (sludge) [g]
$m_{VS,\ substrate}$ – volatile solids mass of substrate [g]

The batch tests were prepared in the following way:

First, the batch test bottles were prepared, for this, the right amount of the plastic hose was cut, so that they can be attached to the batch test outlet valve and to the flow regulation valve. The other piece of hose was connected to the biogas bag, which was connected to the flow regulation valve as well, as can be seen from the Picture 4.

Picture 4. Empty batch test bottles with connected flow regulation valves ready to be filled up

Next, the batch test bottles were filled up with sludge, according to calculated amounts from formulas 13, 14 and 15. Two batch tests were prepared as negative controls (only sludge), two batch tests with distillery waste and cyanobacteria (proportion 80-20%) and the last two were prepared with only distillery waste monosubstrate (precise amounts can be seen from Appendix 3). Each of the batch test bottle caps were attached to the previously tested biogas bags for batch tests.

Nitrogen gas was introduced to the internals of batch test bottles to remove oxygen, so that the anaerobic conditions would be met. First, the batch test bottles were opened, then, vaseline was spread around the batch test bottle caps, afterwards, nitrogen gas was introduced inside the bottles for 10-20 seconds and then the batch test bottle caps were placed as fast as possible back into the batch test bottles, consequently sealing them [26] [27].

All of the prepared batch test bottles were shaken and then transferred into the prepared water bath, which was keeping the water at 42 °C (mesophilic conditions). Picture 5 shows batch tests in operation.

Picture 5. Prepared batch tests. Two bottles on the left are with distillery waste, two in the middle are distillery waste with cyanobacteria and two on the right are negative controls (sludge)

From this step, every day each bottle was shaken once to maintain a better substrate uptake for microorganisms. [28]

3.3 Operating continuous anaerobic reactors

As for the next part of the whole work, the bioreactors (anaerobic digesters) were prepared. The bioreactors that were used for this work were made of plastic material and had the capacity of 12 litres, with attached stirrers from the top (stirred according to the set timer, which was every half an hour), which can be seen from Picture 6. Bioreactor (number 1) itself was heated up by water jacket (42 °C temperature, mesophilic condition)

through the water bath (behind the bioreactors), which allowed the microbiological processes happen. And finally, for the collection of biogas, the aluminum material methane approved gas bags (number 2) were tested and then used for collection of produced biogas.

Picture 6. Bioreactors (left one for distillery waste and right one for cyanobacteria with distillery waste)

In order to achieve anaerobic conditions, the top of the bioreactors was filled up with water (also called water pocket) to prevent air from getting into the reactor. Also, a 50 ml tube attached to a metal stick was used to take samples/remove the excess sludge from the bioreactors. With this tube, the sludge was taken from other bioreactors to fill up the used bioreactors with sludge (final volume must be 12 liters).

Important notice: the automatic stirring must be turn off while removing the contents in the bioreactor! If the stirring activates during removal of the contents, the damages may occur.

3.3.1 Total solids and volatile solids

For the determination of the amount of fresh mass of substrate/inoculate needed for feeding the bioreactors, the volatile solids composition was needed to be determined. For this, first the material that was planned to be used as substrate/inoculate was weighed and then placed to the preheated oven to 105°C for 48 hours. After drying to constant weight, the material was taken out from the oven, placed to desiccator to cool down, weighed on the scales and then placed to the muffle oven at 550°C for 6 hours. Afterwards, the samples were taken out from muffle oven, placed to desiccator once more and then weighed on the scales. [26] [29] [27]

For the determination of total solids (TS) and volatile solids (VS), the following formulas were used [26] [27]:

$$TS\ [\%] = \frac{(m_{dry} - m_{empty})[g]}{(m_{full} - m_{empty})[g]} * 100\% \quad 16)$$

where,

TS – total solids [%]
m_{dry} – weight of crucible after 105°C for 48 hours [g]
m_{empty} – weight of an empty crucible [g]
m_{full} – weight of a full crucible [g]

$$VS\ [\%] = \frac{(m_{dry} - m_{ash})[g]}{(m_{full} - m_{empty})[g]} * 100\% \quad 17)$$

where,

VS – volatile solids [%]
m_{dry} – weight of crucible after 105°C for 48 hours [g]
m_{ash} – weight of crucible after 550°C for 6 hours [g]
m_{empty} – weight of an empty crucible [g]
m_{full} – weight of a full crucible [g]

In order to have more precise results, triplicates of the sample were made for each time volatile solids determination was performed. Therefore, the average was taken from the triplicates by using formula 18 [26] [27]:

$$\frac{x_1 + x_2 + x_3}{3} = \overline{m_x} \qquad 18)$$

where,

x_1,x_2,x_3 – values of each sample
$\overline{m_x}$ – mean value of the samples

The calculate values can be seen from Appendix 4.

3.3.2 Analysis of substrates

3.3.2.1. Corn

The first substrate that was used for bioreactor operation was corn. One of the crucial parts before feeding the substrate to the reactors, was to identify the total solids of the planned substrate (more about this in the "Preparation stage" section). Nevertheless, this step was bypassed in this work, since the information about total solids of corn was already identified by other users of this substrate. Therefore, it was straight away possible to identify the amount of fresh mass of substrate that was needed to be fed to the bioreactors by simple calculations mentioned in section "Loading rate".

3.3.2.2. Distillery waste

As for the second substrate, the task was to find the distillery waste from the nearest breweries/distilleries in Lower Saxony, Germany. After contacting several places, it was possible to receive the substrate from Wöltingeröde. The first batch of distillery waste was 60 liters (Picture 7).

Picture 7. Two barrels of distillery waste from Wöltingerode

Three crucibles were taken, weighed on the scales, and then each was filled up with 100 g of distillery waste. Afterwards, the volatile solids content was identified by following the method described in the "Total solids and volatile solids". Later, due to fouling situation with the first batch, the second batch was taken from the same place and was analysed the same way. The substrate was then separated into 1 litre portions and stored at -18°C (Picture 8).

Picture 8. Distillery waste separated into 1 litre portions

3.3.2.3. Preparation of co-substrate

The co-substrate of this bachelor thesis was cyanobacteria (*A. platensis*), that was collected from the photobioreactors during harvesting. Each time, when harvesting the cyanobacteria biomass, it was waited for 10 minutes after the water level was not seen in the sieve, making it possible to have a similar water content of cyanobacteria biomass after each harvest cycle.

Since there was already some cyanobacteria biomass collected in the previous harvest cycles before this bachelor work, the previously harvested amount was thawed and then mixed in a large beaker with the new harvested amount, as can be seen from Picture 9 (the total amount of the mix was around 2800 g). Around 300 ml was taken from the mix of the whole cyanobacteria biomass and then three crucibles were weighed, and each was filled up with 50 g of cyanobacteria biomass (the residual 150 ml was put back to the large beaker). The volatile solids content was identified by following the method in the "Total solids and volatile solids" sub-chapter.

Picture 9. Thawed cyanobacteria biomass mixed together

Afterwards, the cyanobacteria biomass mix was distributed into separate portions and then kept at -18 °C.

3.3.3 Loading rate and fresh mass of substrate

Loading rate is a factor which indicates the amount of substrate that is needed to be fed according to organic matter of the material (VS).

The fresh mass amount of each different substrate was needed to be calculated with the following formulas [26] [27]:

$$0{,}5\ \frac{g\ VS * \ V_{fermenter}[L] * Time\ [d]}{L * d} = VS\ [g] \qquad 19)$$

where,

$V_{fermenter}$ - volume capacity of the fermenter [L]
t – time [d]
VS – volatile solids mass of substrate [g]

$$m_{substrate}\ [g] = \frac{VS\ [g] * 100\%}{VS_{Substrate}} \qquad 20)$$

where,

$m_{substrate}$ – fresh mass of the substrate [g]
VS – volatile solids mass of substrate [g]
$VS_{substrate}$ – volatile solids of the substrate [%]

From the calculations, $m_{substrate}$ was the indicative value which showed how much substrate was required to be fed of the material in the question.

For the calculation of cyanobacteria-distillery waste mix, same formulas (19, 20) were used. Since cyanobacteria composition was 20%, the calculated mass of substrate from formulas was multiplied by 0,2 (20% of the 100% composition), and for distillery waste 80% were calculated by multiplying the calculated mass of substrate by 0,8. The calculated proportions were summed, thus having total mass substrate of cyanobacteria-distillery waste mix.

3.3.4 Feeding the bioreactors

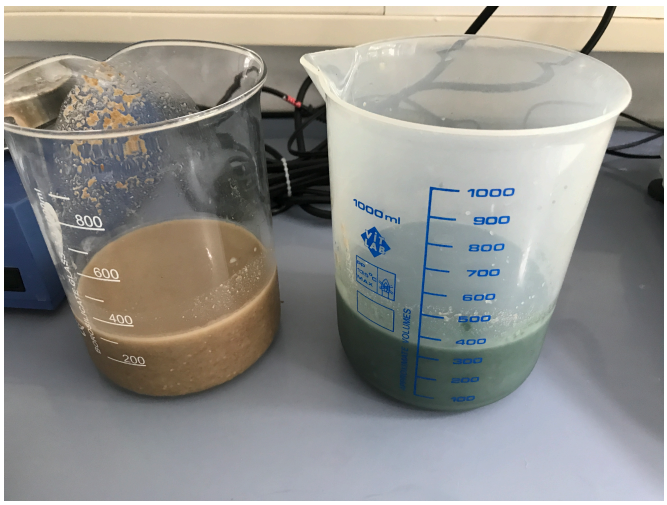
The fresh mass of substrate was calculated according to loading rate and added to appendix 5.

3.3.4.1. Corn

When fed with substrate, corn was weighed on scales according to the calculated loading rate value and then transferred through the funnel into the bioreactor. For this, stirring was turned off and the top plug in the bioreactor was removed. Amount of sludge from the reactor was same mass removed as the mass of substrate fed, thereafter the reactor was closed with the plug. In the end, stirring was turned back on.

3.3.4.2. Distillery waste and cyanobacteria-distillery waste mix feed

First of all, the substrate was heated up. When feeding with distillery waste, the required amount of the substrate was weighed on the scales according to loading rate calculated. The feeding was done in the similar way as feeding with corn, except the funnel was not necessary to be used. For the distillery waste and cyanobacteria mix, first calculated amount of distillery waste was weighed in a beaker, then the calculated amount of cyanobacteria biomass was transferred into the same beaker, as can be seen from Picture 10. Before feeding, the stirring was turned off and the approximate amount of sludge from bioreactor proportional to the amount of substrate was taken out from the bioreactor and disposed accordingly. The prepared substrate was added to both bioreactors, and the plug was closed to continue the microbiological processes in the fermenter. Of course, after feeding, the stirrers were required to be turned back on.



Picture 10. On the left, distillery waste and on the right distillery waste and cyanobacteria mix ready to be transferred to bioreactors

3.3.5 Substrate composition

The composition of distillery waste was according to Mr. Daeger 90 % wheat, 10 % barley lard with alcohol content before brewing 9 % and after brewing 0,2 %. The dry content estimated by Mr. Daeger was 10,4 % with 5,39% nitrogen, 2,48 % phosphorus pentoxide (P_2O_5), 1,57 % potassium oxide (P_2O), magnesium 0,56 % and sulfur 1,57 %. The pH of this material was 3,99. The color of the material was beige. As from personal analyses, the first distillery waste that was taken had VS 10,77 % and the second one had 7,77 %.

For cyanobacteria biomass, it was *Arthrospira platensis* specie, from which VS were identified as 6,37 % and pH of this material was 10,18. The color of the material was dark green.

3.4 Taking biogas measurements

The biogas measurements from bioreactors and from batch tests were carried out the same way. First, the outlet pipe of the gas was put outside of the window, then the biogas bag with biogas was connected to the inlet pipe. Before measuring the gas, values of current time, temperature and pressure were taken from the device in the room.

The starting values of the volume were taken from the compressor (Ritter) and the device that measures the biogas composition (SR2-DO) was turned on to analyse the gas (see Picture 13). The gas was flowing through the biogas composition measuring device to measure CH_4, CO_2 and H_2S. It was necessary to wait for around 2-3 minutes to get the constant values for these compounds, then the values were noted down. Afterwards, when done measuring the biogas composition, the valves were turned so that the gas would flow through the pump. Pump was useful, because it empties the biogas bags much faster, thus speeding up the process. When the biogas bag was empty, the volume values were noted down again from the compressor. The difference between the start of measurement and end of measurement was the biogas volume inside the biogas bag.

Picture 13. 1) Compressor (Ritter), 2) pump, 3) gas composition measuring device (SR2-DO)

The noted values from biogas measurements were then needed to be converted to norm liters of biogas. For this, Formula 21 was used [26] [27]:

$$V_{0,biogas}{}^{dry}[L] = V\,[L] * \frac{(p - p_w)[mbar] * T_0[K]}{p_0[mbar] * T[K]} \quad 21)$$

where,

$V_{0,biogas}{}^{dry}$ – norm liters of biogas, dry [L]
V – total collected volume [L]
p – atmospheric pressure [mbar]
p_w – water pressure at the current atmospheric temperature [mbar]
p_0 – normal pressure, 1013,15 mbar
T – atmospheric temperature during gas measuring [K]
T_0 – norm temperature [K]

Please note, that p_w value was identified according to the temperature of the time of measurement, by using the literature values. If the temperature was between the required values, interpolation of the exact p_w value was necessary. The literature values that were necessary for this work can be seen from Table 2.

Table 2. p_w values for specific temperatures in mbar [30]

t/°C	0	2	4	6	8	10	12	14	16	18
0	6,112	7,060	8,135	9,353	10,729	12,281	14,027	15,989	18,187	20,646
20	23,392	26,452	29,857	33,638	37,809	42,452	47,582	53,240	59,472	66,324

As for the determination of the norm concentration of methane and norm liters of methane, following formulas were used [26] [27]:

$$C_{dry}[\%] = C_{moist} * \frac{p}{p - p_w} \quad 22)$$

where,

C_{dry} – dry concentration of methane [%]
C_{moist} – moist concentration of methane [%]
p – atmospheric pressure [mbar]
p_w - water pressure at the current atmospheric temperature [mbar]

$$C_{dry,corr}[\%] = C_{dry,2}[\%] + ((C_{dry,2} - C_{dry,1}) * \frac{V(H)}{V_{0,Biogas}{}^{dry}})[\%] \quad 23)$$

where,

$C_{dry,corr}$ – corrected methane concentration [%]
$C_{dry,2}$ – methane concentration after current measuring [%]
$C_{dry,1}$ – methane concentration after the previous measuring [%]
V (H) – volume of the head space [L]
$V_{0,biogas}{}^{dry}$ – norm liters of biogas, dry [L]

Afterwards, norm liters of methane can be calculated with Formula 24 [26] [27]:

$$V_{methane}[L] = C_{dry,corr}[\%] * V_{0,Biogas}{}^{dry}[L] * \frac{1}{100}\% \quad 24)$$

where,

$C_{dry,corr}$ – corrected methane concentration [%]
$V_{0,biogas}{}^{dry}$ – norm liters of biogas, dry [L]
$V_{methane}$ – norm liters of methane [L]

In the end, the calculated biogas values were converted to methane/biogas production per kg VS by using Formula 25 [26] [27]:

$$\frac{V_{methane}[L]}{m_{VS,substrate}[kg]} = V_{methane}[L] * \frac{1000\ \left[\frac{g}{kg}\right]}{m_{VS,substrate}[g]} \quad 25)$$

where,

$V_{methane}$ – norm liters of methane [L]

$m_{VS,substrate}$ – volatile solids mass of substrate [g]

The calculated values were based on the data that can be seen from Appendix 6 (for batch tests) and Appendix 7 (for continuous bioreactors), and were the bases for making graphs in the "Results" chapter.

3.5 Testing biogas bags

From the Tables 3, 4 and 5, data from biogas bags can be seen. The bags were tested by inflating the known amount of air, placing some weight on the bags and leaving them overnight. Then, bags were deflated and the differences in the air volume can be seen. By discussing the results with practical thesis supervisor, it was agreed that the bags are tight and can be used for collecting biogas.

Table 3. Data recorded when testing biogas bags for continuous reactors

	Inflate		Deflate	
	Bag 7	Bag 5	Bag 7	Bag 5
Before measuring (L)	815	875	940	0
After measuring (L)	875	935	999,84	58,75
Difference (L)	60	60	59,84	58,75

Table 4. Data recorded when testing smaller biogas bags for continuous reactors

	Inflate		Deflate	
	Bag 1	Bag 2	Bag 1	Bag 2
Before measuring (L)	88,5	94,2	100,5	106,5
After measuring (L)	94,2	100,35	106,5	112,4
Difference (L)	5,7	6,15	6	5,9

Table 5. Data recorded when testing bags for batch tests

	Inflation					
	Bag 1	Bag 2	Bag 3	Bag 4	Bag 5	Bag 6
Before measuring (L)	302,5	308,5	314,5	326,5	332,5	338,5
After measuring (L)	308,5	314,5	320,5	332,5	338,5	344,5
Difference (L)	6	6	6	6	6	6
	Deflation					
	Bag 1	Bag 2	Bag 3	Bag 4	Bag 5	Bag 6
Before measuring (L)	423,3	429,25	435,15	441,1	447	452,95
After measuring (L)	429,25	435,15	441,1	447	452,95	458,9
Difference (L)	5,95	5,9	5,95	5,9	5,95	5,95

3.6 FOS/TAC and ammonium-nitrogen analysis

Twice per week the contents in the bioreactors were analysed to see pH, ammonium nitrogen (NH_4-N), FOS/TAC ratio and single acid concentration (next sub-chapter).

First, around 50 ml of the sludge was taken from both reactors and the required amount (usually around 25 ml) of it was weighed to be put to centrifuge for 20 minutes at 10000 rpm. Afterwards, 5 g of supernatant was transferred to the beaker and filled up with Millipore water until the analytic balance showed 20 g. The beakers were then put on the stirrer, where pH meter and titration instrument (with 0,5 M H_2SO_4) were put inside the beaker. Then, H_2SO_4 was added to the beaker, until the pH meter showed pH 5.00. The values were noted down and more acid was added to the beaker until pH showed 4.40. With the amount of added acid, it was possible to calculate FOS and TAC values from the formulas 26 and 27. [26] [31] [27]

$$FOS[mL] = 250 * \frac{20g}{5g} * amount\ of\ 0{,}5M\ H_2SO_4\ until\ pH\ 5.00 \quad (26)$$

$$TAC[mL] = \left(\left((amount\ of\ 0{,}5M\ H_2SO_4\ until\ pH\ 4.40\ * \frac{20g}{5g} * 1{,}66\right) - 0{,}15\right) * 500\right) \quad (27)$$

When the FOS and TAC values were calculated, FOS was divided by TAC, then the condition of the bioreactor regarding feeding was checked from Table 6 with the calculated ratio. [26] [31] [27]

Table 6. Condition of the reactor regarding feeding [26] [27]

FOS/TAC value	Condition	Action
>0,6	Reactor is very overfed	Stop feeding immediately
0,5-0,6	Reactor is overfed	Reduce feeding
0,4-0,5	Reactor is slightly overfed	Increase process control
0,3-0,4	Reactor is optimally fed	Pause feeding
0,2-0,3	Reactor is slightly underfed	Slowly raise feeding
<0,2	Reactor is very underfed	Increase feeding fast

As for NH_4-N determination, the following tests were carried out. 1 mL of supernatant from each of the same centrifuged samples was taken and filled up to 30 mL, so that the sample will be diluted 1:30 with Millipore water. 0,2 mL of the diluted sample was added to ammonium test vials (HACH LANGE 302 LCK), caps were turned other way around and shaken gently so that the chemicals from the cap would be dissolved. Then, it was necessary to wait 15 minutes before measuring the NH_4-N concentration. When 15 minutes passed, both samples were measured in spectrophotometer and the concentrations of the samples were multiplied by 30, because the samples were diluted to factor 1:30. [26] [32] [27]

3.6.1 Single acid determination

For single acid determination, around 2 mL of 10% H_2SO_4 was added to the beaker containing 20 g of sludge from bioreactor. When the acid was added, the pH was observed. When the pH was between 1.00 and 2.00, the sample was transferred to the centrifugation tube and placed to centrifuge for 20 minutes at 10000 rpm. [26] [27]

After centrifugation, supernatant was pipetted and then passed through a 0,2 µl filter into a GC-MS vial (VWR company). The vials were then stored in the fridge, until it was possible to analyse these vials with GC-MS for the single acid composition. [26] [27]

4 RESULTS

4.1 Growth curves of the loops

As it can be seen in Figure 3, from the beginning of the work, two loops were set to operate (loop 3 and loop 4), where afterwards, one more loop was started (loop 2). All of these loops had the same culture.

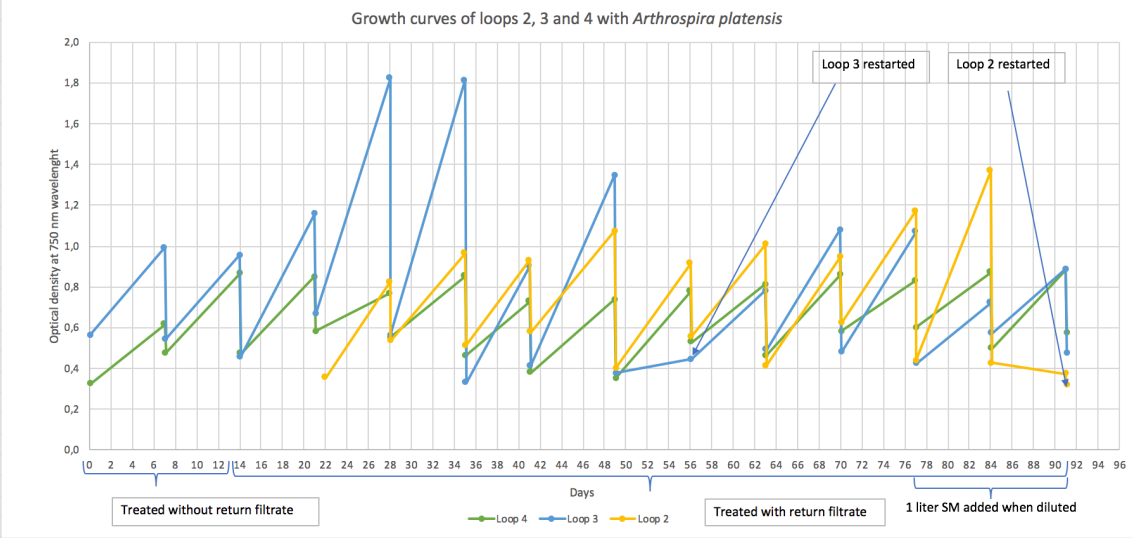


Figure 3. Differences in growth of Arthrospira platensis in the standard medium from loops (2, 3 and 4)

From Figure 3 it can be seen that the growth of the cyanobacteria was highest after it was treated with the return filtrate, especially highest growth was found in loop 3. The conditions of these loops were close to each other, (which can be seen from Materials and Methods chapter "Operating photobioreactor").

As for the filtrate, at the beginning of the experiment, it was used to be discarded completely, although later on, in order to save chemicals and not to waste the medium that might still have not depleted all the nutrients for cyanobacteria, around 90% of collected filtrate was returned back to the loop. In order to reimburse for the 10% that was discarded of the filtrate, the standard medium solution was prepared and added to the loops which were recently harvested. Afterwards, as can be seen from Figure 3, 1 litre of standard medium was added and then the loop was filled up with return filtrate until it was full; the rest return filtrate was discarded. The shift to the new technique was necessary, because during previous way of treating the loops it was not always possible to add the same volume of standard medium when refilling the loop.

During the total experiment time, loops were restarted twice, as can be seen from Figure 3. When restarted, inoculate from operating loops was taken, inoculating the new loop and completing the volume with prepared standard medium.

In the next sub-chapter, the comparability of liquid digestate loop can be seen with the current loops that were discussed.

4.1.1 Digestate loop with *Arthrospira platensis*

In order to see the suitability of liquid digestate as the nutrient source of *Arthrospira platensis* there was a loop that was operated according to the research paper by Hultberg et al. (2016) [33]. By following this paper, a 10 liter loop was started with *Arthrospira platensis* as inoculant from the 20 liter loop (4,925 liters), carbonate buffer (4,925 liters) and liquid digestate (0,150 liters). The carbonate buffer was prepared with a concentration of $NaHCO_3$ (13,6 g/L) and Na_2CO_3 (4,0 g/L), pH 9,2. As for digestate, the required amount of digestate was taken from the storage with digestate and put to centrifugation bottles. The samples were centrifuged with 15000 rpm for an hour.

After the centrifugation, the supernatant from the samples was transferred to the glass bottles, which were then used as containers for liquid digestate. These glass bottles with liquid digestate were autoclaved (120°C). The liquid digestate was the component that was used for preparing the digestate loop, and the addition of such was at first 1,5 % (v/v), after 4 days 1,5 % (v/v) and after 7 days 3 % (v/v) (which is considered the final addition according to the research paper by Hultberg et al. (2016) [33]). Afterwards, 1,5% (v/v) was added again to the loops (on day 41), to see if this would improve the growth rate. The growth rate of digestate loop can be seen from Figure 4.

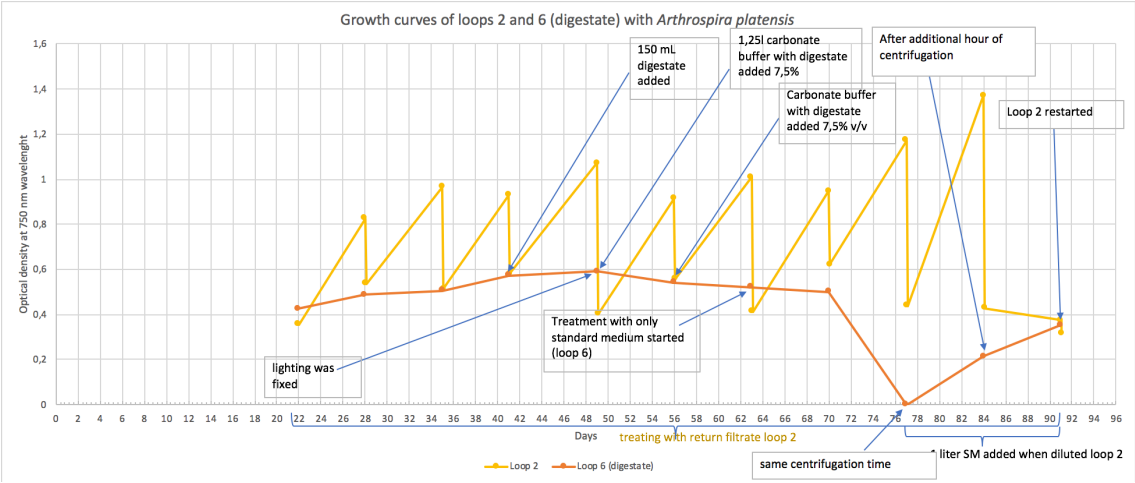


Figure 4. Suitability of liquid digestate for cultivation of A. platensis

From the Figure 4, the comparison between a digestate and non-digestate loop with the same culture can be seen. The reason why the graphs start from day 22, is because these loops were put into operation later than the previous two. It can be seen, that there was almost no growth in the digestate loop, while loop 2 was growing very actively. Especially, it can be seen that at some point that there is a steady decline in the cells. In order to treat such a steady decline, instead of digestate and carbonate buffer, standard medium was added from day 77. This has led to an increased growth (although with a higher centrifugation time). The lighting was not working in digestate loop (only noticed on day 49, and fixed the same day), but later on the growth of cells was still not seen.

Additionally, the daily growth rate of each loop per week was calculated and added to Figure 5.

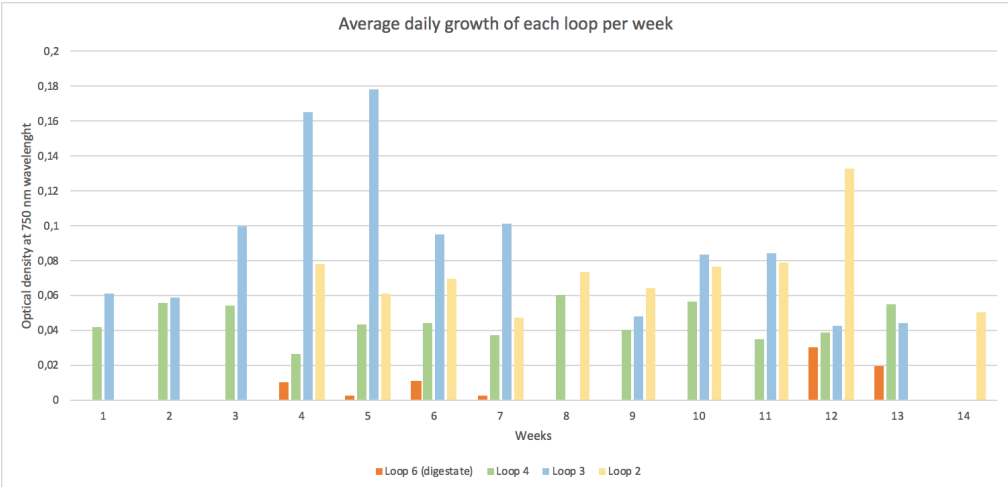


Figure 5. Average daily growth of Arthrospira platensis of each loop per week

From the Figure 5, it is possible to see that the highest growth was measured from loop 3 (blue), whereas the lowest growth was seen from digestate loop (yellow). Please also note, that the loop 3 was restarted on week 8, and loop 2 was restarted on the last week of the experiments.

As well, the recorded biomass of *A. platensis* was calculated from loops 2, 3 and 4, as a representation of biomass collected from each liter of harvested medium. The data can be seen from Figure 6. As for the biomass collected from digestate loop, the amount was recorded to be around 25 g after the entire time of operating (which is the reason it was not included in the Figure 6).

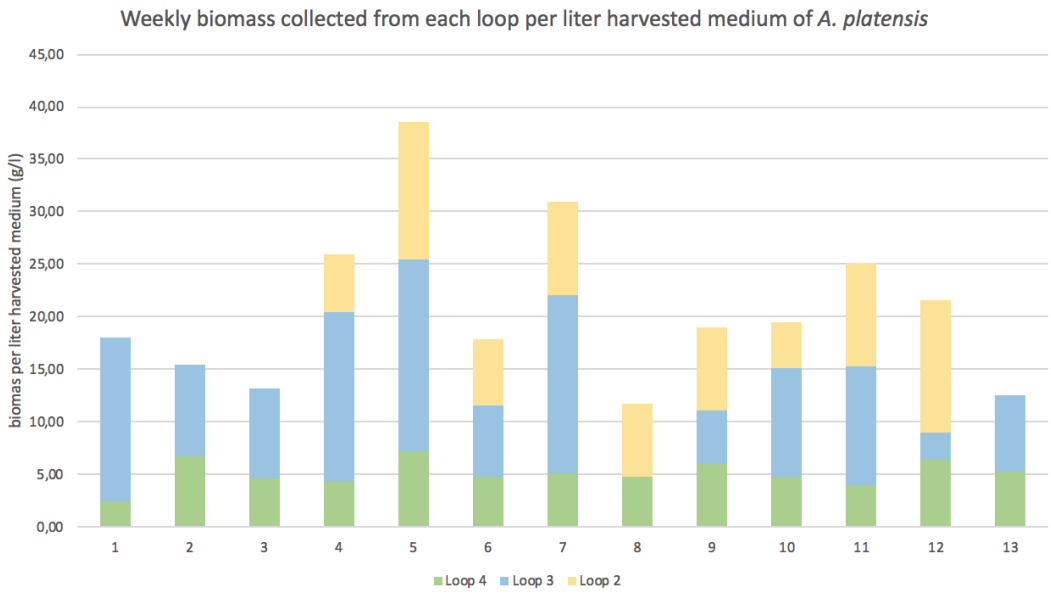


Figure 6. Representation of Arthrospira platensis biomass collected from each week in grams per liter for each loop

From Figure 6, the highest amount of biomass collected was recoded on week 5 and week 7. As it can be seen, loop 3 was the most productive during the total experiment time. The highest amount of *Arthrospira platensis* biomass that was possible to get from loop 3, was 18,39 g/l of harvested medium (week 5), while the second highest amount of biomass was seen in loop 2 (13,06 g/l of harvested medium for week 5 as well).

With the collected biomass from the loops, it was possible to use portions of it for anaerobic digestion, results of which can be seen from the next sub-chapters.

4.1.2 Microscopy of loop contents

The loops were checked under microscope at magnification 400x. The pictures of them can be seen from Appendixes 8, 9, 10, 11.

From the Appendix 8, it can be seen that loop 2 with *Arthrospira platensis* is contaminated and the cells are under stress (measured on day 90). Later on, there was a sudden decline of the culture, that has happened on day 91.

As from Appendix 11, it can be seen that the culture is darker because liquid digestate was used. However, it was possible to see cells present with the microscope, meaning that the decline of cells was not the issue, but rather the methodology of measurement should have been adjusted.

4.2 Batch test results

The batch tests were operated under mesophilic conditions (42°C). The biogas and methane potentials were measured for the same substrates that are used for continuous anaerobic digestion (distillery waste and cyanobacteria-distillery waste mix). The reason why it was necessary, was because of the differing sludge in both continuous bioreactors (which could have an influence on the results).

The batch test biogas bags from substrates were emptied on days 1, 4, 6, 13, 35. While the biogas bags from negative controls were emptied on day 35.

4.2.1 Results from negative control batch tests

From Figure 7, it is possible to see the amount of norm litres biogas and methane produced from negative controls (sludge) during the whole time of batch test (35 days).

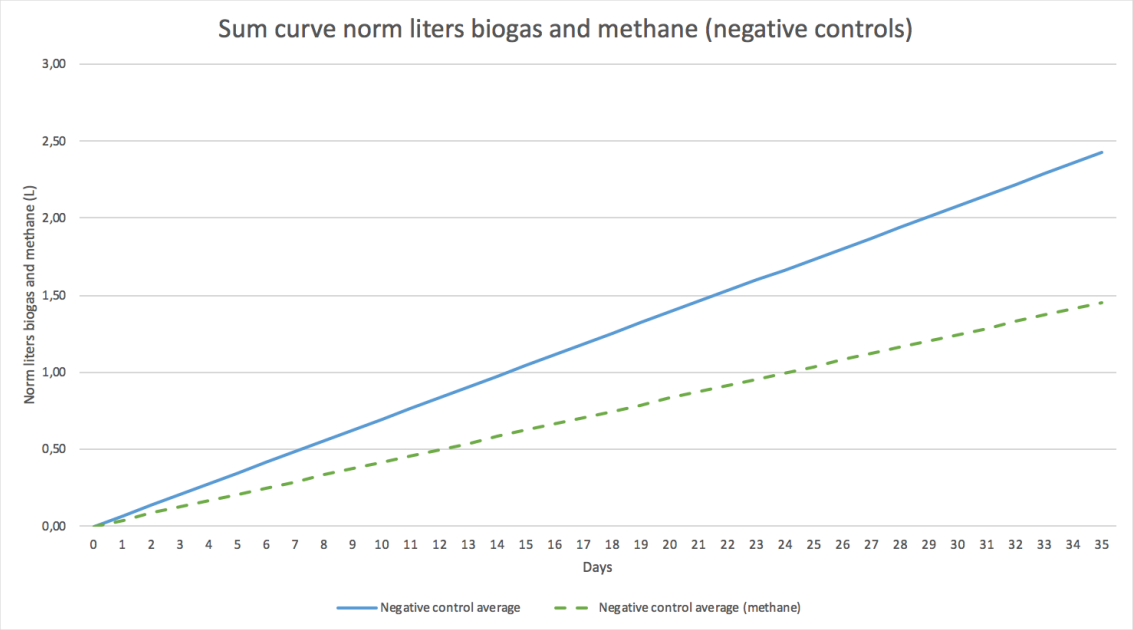


Figure 7. Sum curve of norm liters biogas and methane for negative controls (sludge) from batch tests (averaged values of two duplicates)

The values from negative controls were subtracted from the values collected for distillery waste and cyanobacteria-distillery waste mix substrates. Therefore, the following graphs for those substrates already show the actual values. The recorded methane concentration was around 60% in average from both duplicates.

4.2.2 Sum curve of biogas and methane production

Please note that the lower value on day 4 is the result of a fast evaporation of water, which lead to a shutting down of the water bath, thus not keeping the water in mesophilic condition (42°C). Afterwards, this issue has never happened again.

From Figures 8 and 9 The batch test results can be seen. From the graph, highest biogas results were found from distillery waste II, while according to methane content, the volume of methane gas was similar to distillery waste I.

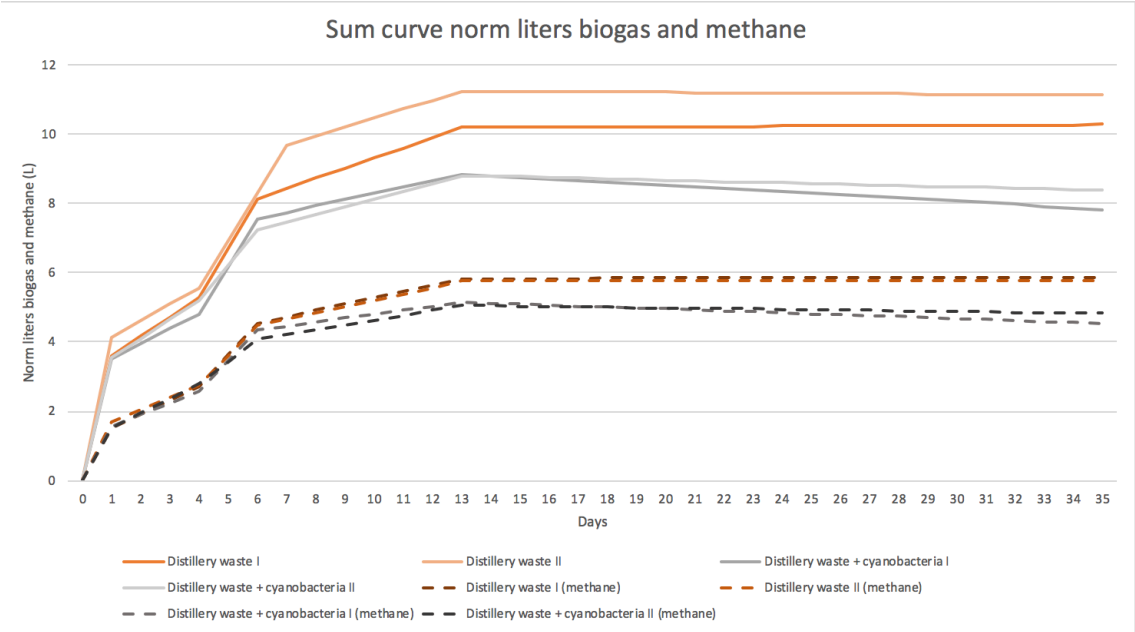


Figure 8. Influence of Arthrospira platensis on the biogas/methane potential of distillery waste

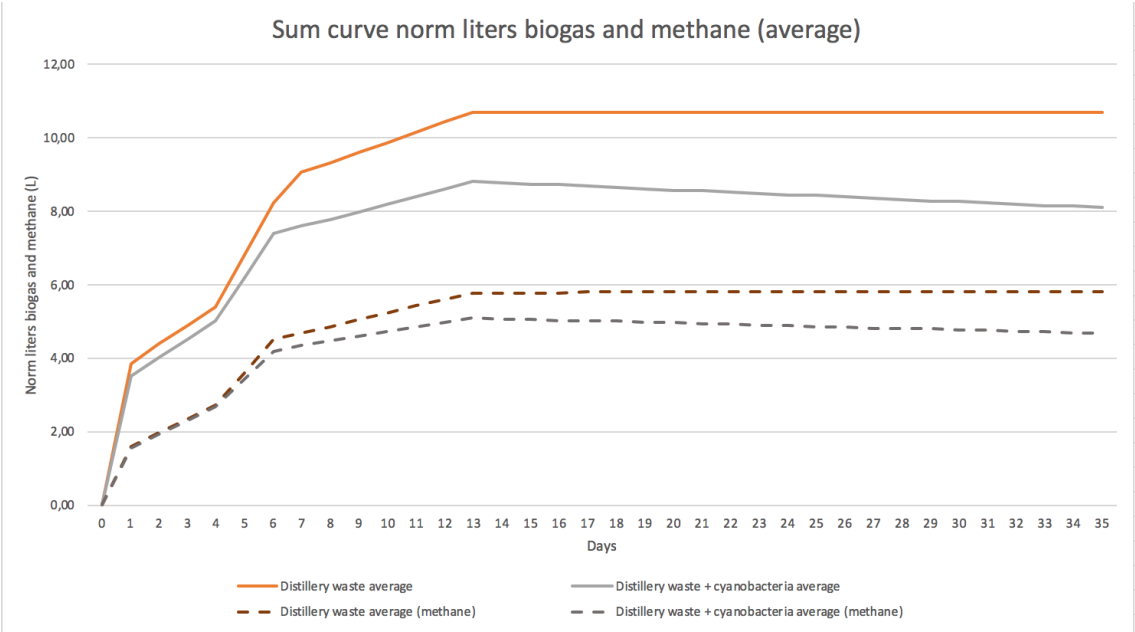


Figure 9. Influence of Arthrospira platensis on the biogas/methane potential of distillery waste (average)

From Figure 9, the average results from duplicates showed that distillery waste had a higher biogas and methane production (10,70 l biogas and 5,83 l methane) than of cyanobacteria-distillery waste mix (8,11 l biogas and 4,69 l methane), which is around 24 % difference for biogas and around 20 % difference in methane.

4.2.3 Sum curve of biogas and methane production per kg VS

From the Figures 10 and 11, the graph looks similar to the previous ones, except this time the biogas and methane production is compared to kg VS. Here, the methane content of both different substrates seems to be closer.

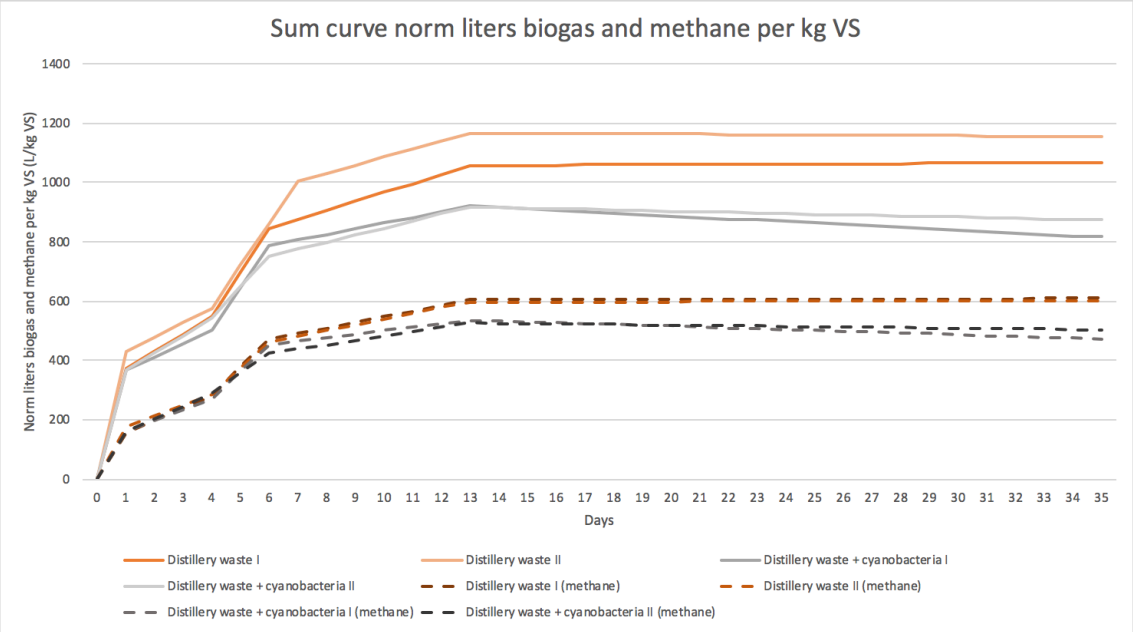


Figure 10. Influence of Arthrospira platensis on the biogas/methane potential of distillery waste (l/kg VS)

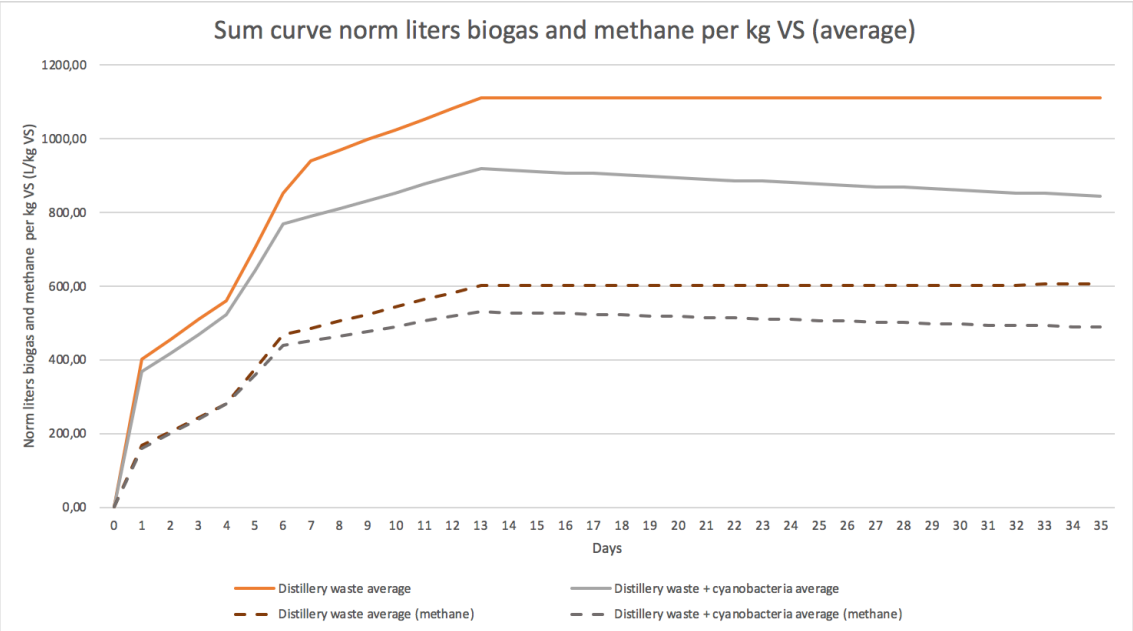


Figure 11. Influence of Arthrospira platensis on the biogas/methane potential of distillery waste (average, l/kg VS)

From Figure 11, the average results from distillery waste duplicates showed 1111,14 l biogas/kg VS and cyanobacteria-distillery waste mix showed 844,70 l biogas/kg VS, which is 24 % lower than distillery waste monosubstrate. For methane production per kg VS, the difference is approximately 20 %.

4.2.4 Sum curve of biogas and methane production per kg fresh mass

As from the Figures 12 and 13, the biogas and methane content were compared to kg fresh mass substrate used. Also, as previously, distillery waste I differs from distillery waste II, while the methane content is similar. Overall, the methane content for both substrates is similar.

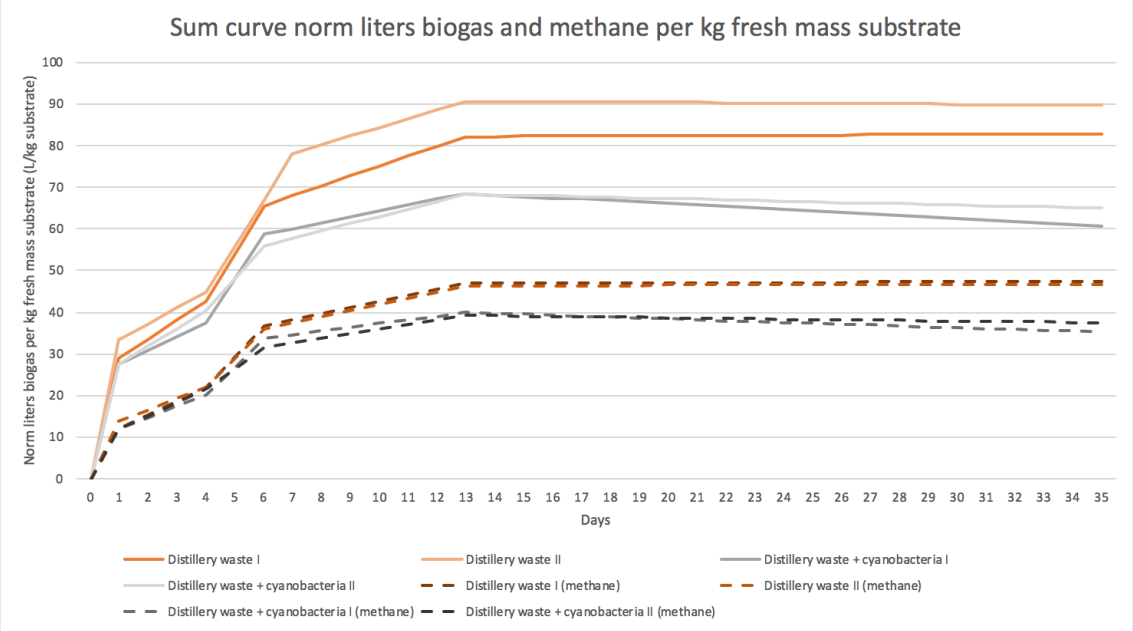


Figure 12. Influence of Arthrospira platensis on the biogas/methane potential of distillery waste (l/kg fresh mass substrate)

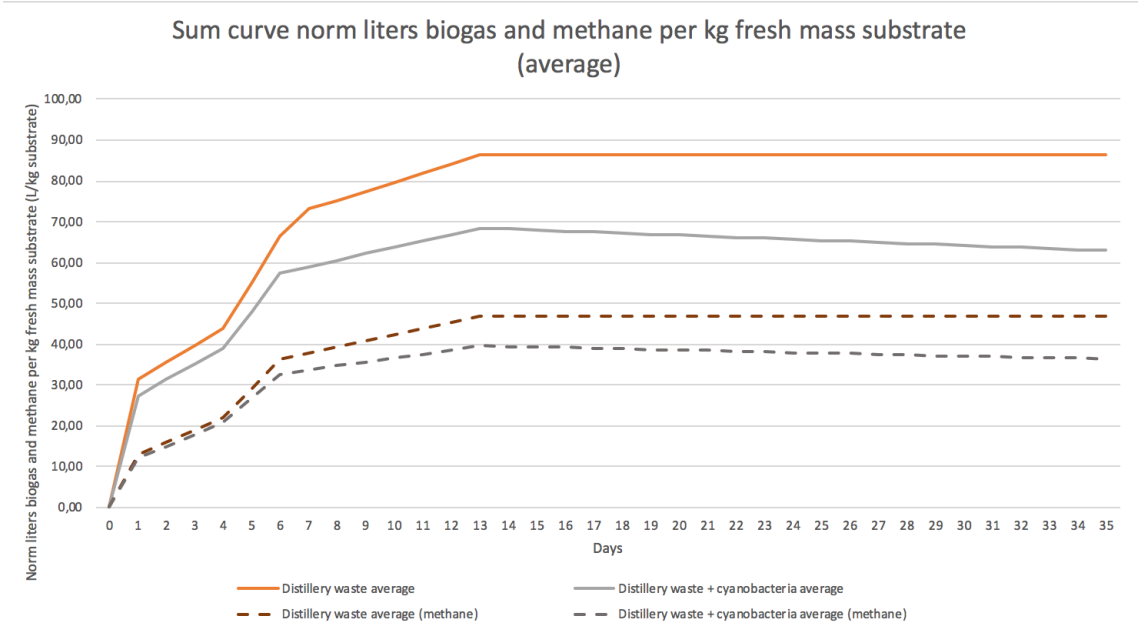


Figure 13. Influence of Arthrospira platensis on the biogas/methane potential of distillery waste (average, l/kg fresh mass substrate)

From Figure 13, the average results from distillery waste substrate duplicates showed 86,34 l biogas/kg fresh mass substrate and cyanobacteria-distillery waste substrate showed 62,91 l biogas/kg fresh mass substrate, having distillery waste substrate approximately 27 % higher. As for methane production, distillery waste was 47,03 l methane/kg fresh mass substrate and cyanobacteria-distillery waste substrate 36,36 l methane/kg fresh mass substrate (approximately 23 % lower than distillery waste).

4.2.5 Concentrations of H_2S, CO_2 and CH_4 corrected

According to Figure 14, at the beginning hydrogen sulphide concentration is higher from the mix of cyanobacteria and distillery waste substrate. The corrected methane concentration is similar (cyanobacteria with distillery waste highest), with a value close to 70 % and CO_2 concentration is lowest for cyanobacteria with distillery waste (35 % at day 4). Afterwards, there was no hydrogen sulphide present in the tests and the average methane concentration between two substrates was around 60 %, having cyanobacteria-distillery waste mix substrate as the highest (61 %, higher than distillery waste by 2 %). CO_2 concentrations were similar by the end of experiment (approximately 39 %).

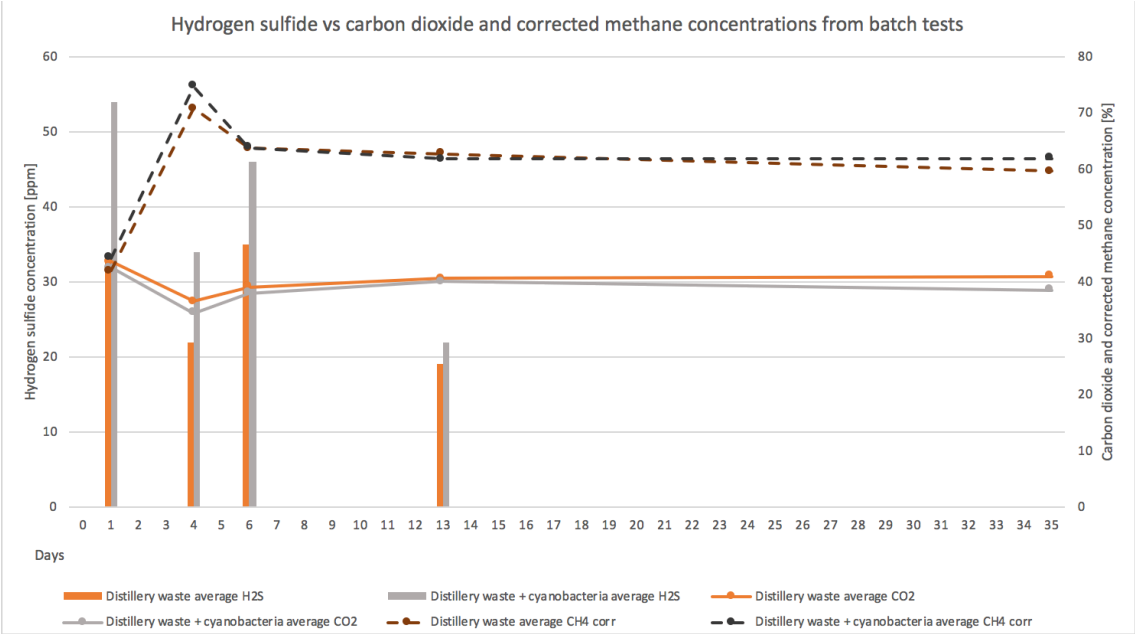


Figure 14. Influence of Arthrospira platensis on the CO_2, CH_4 and H_2S concentrations of distillery waste (average)

4.3 Biogas results from continuous reactors

For biogas results, distillery waste and cyanobacteria-distillery waste mix substrates were tested. By feeding daily continuous anaerobic reactors with these substrates, every next day the biogas bags were emptied, and the results were written down. From the collected results, it was possible to see the biogas potentials of these substrates, which can be seen from the next sub-chapters.

4.3.1 Biogas and methane production per day

Figure 15 describes the liters of biogas and methane produced, which might not be very informative in comparison to other types of substrates which are available from the literature. Nevertheless, with Figure 15 it is possible to see how much biogas was produced throughout the whole experiment.

The mentioned figure is the basis for calculating the norm liters of produced biogas and methane for the next graphs (Figure 16 and Figure 17, in the next sub-chapters).

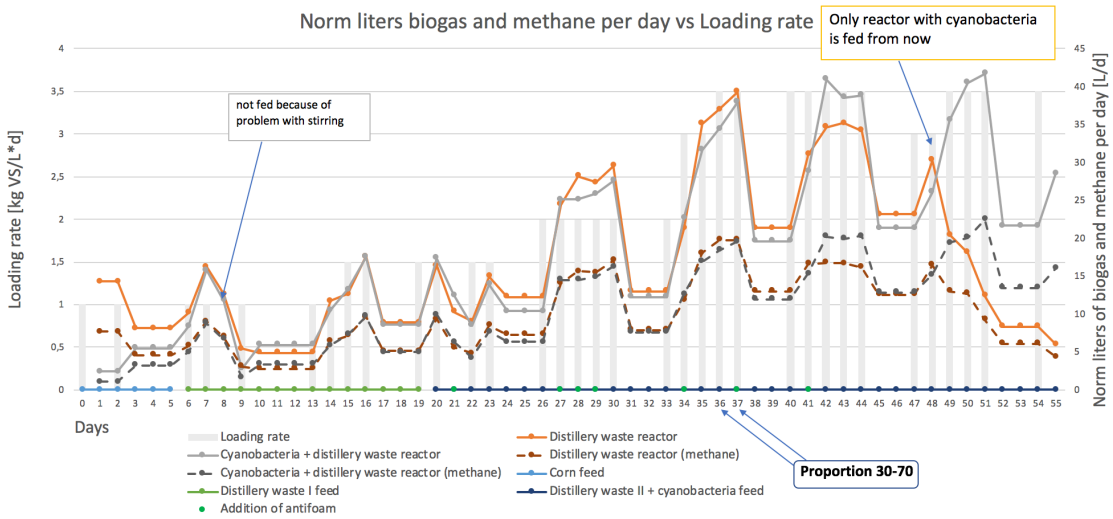


Figure 15. Norm liters biogas and methane production per day against loading rate from two continuous bioreactors

4.3.2 Biogas and methane production per day per kg VS

From Figure 16, first substrate which was fed is corn, next is distillery waste and finally one reactor fed with distillery waste and other with cyanobacteria-distillery waste mix (proportion 20-80). We can see an instantaneous increase of biogas production to the highest value when the corn substrate was changed to distillery waste (at day 7). The methane concentration of it dropped by 3,5 %, which does not necessarily interfere with the production increase of 400 norm litres methane per kg VS compared to results from corn substrate. Afterwards, the graph goes with ups and downs, according to the loading rate, although later on, the productivity of biogas does not increase anymore once the reactors are fed with loading rate 3,5 (also, the decrease in methane gas can be observed). Also note, that the reactor with cyanobacteria-distillery waste mix was fed with 30-70 proportion, but then reduced back to 20-80 proportion due the reasons of availability of feeding material.

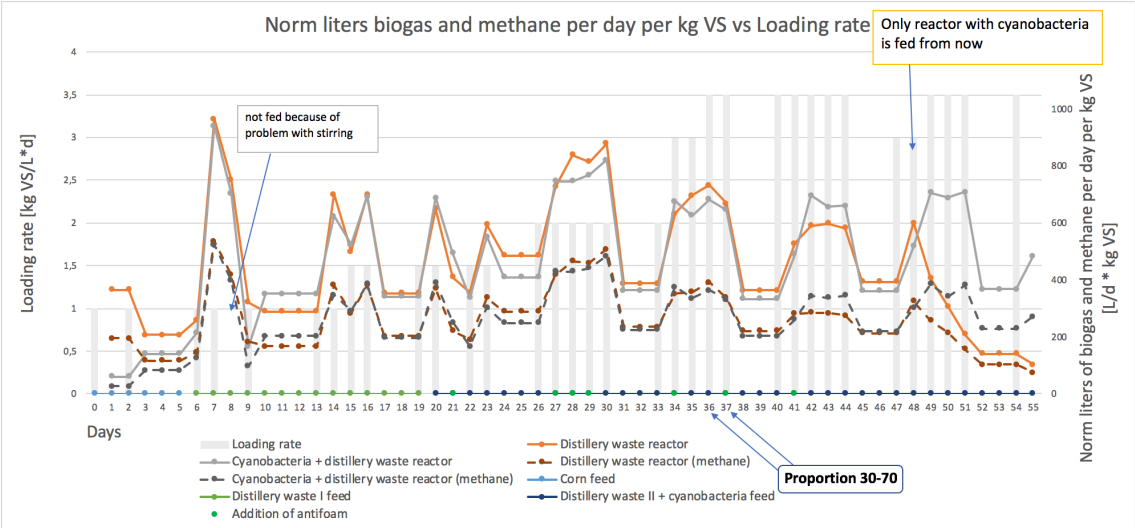


Figure 16. Norm liters if biogas and methane per day per kg VS against loading rate from two continuous bioreactors

4.3.3 Biogas and methane production per day per kg fresh mass

Here, from Figure 17 the development of biogas and methane can be seen according to the amount of fresh mass fed. The same as in the previous figure, the rapid increase of biogas and methane can be seen when the corn is switched to distillery waste, but day earlier (day 6, instead of day 7). Afterwards, the higher values can be seen at days 15 and 16 (highest around 84 l biogas and around 45 l methane per kg fresh mass substrate for day 15), when the reactors were still fed with only distillery waste. Although, later on, the biogas and methane production were not increasing, but keeping at the same level, despite of increased loading rate.

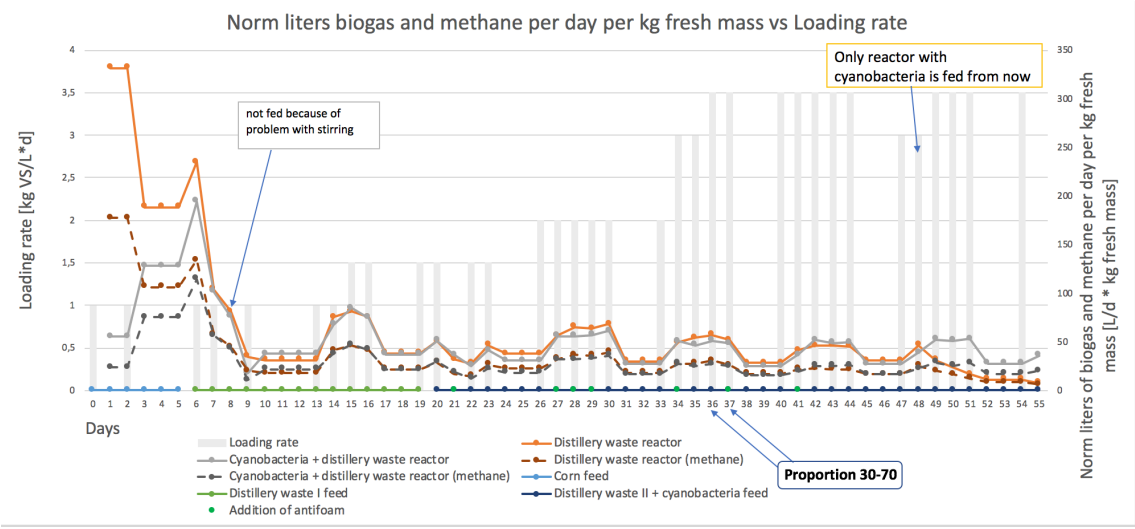


Figure 17. Norm liters biogas and methane per day per kg fresh mass substrate against loading rate from two continuous bioreactors

Please note that on day 21 highest amount of silicone antifoam solution was added to reactor with distillery waste only (4 ml) to reduce foaming problem, while other days (27, 28, 29, 34, 37 and 41) only 1-1,5 ml were added to both reactors, when the foaming problem could not be resolved with stirring. No substantial interference was observed from the biogas results regarding the antifoam addition.

Also, please note that the distillery waste was changed at day 20, to the one which had a 3% lower VS content. Which is why the fresh mass of substrate feed has increased.

4.3.4 Concentrations of H_2S, CO_2, CH_4 dry, NH_4-N and FOS/TAC

Additional parameters were necessary to be analysed from the bioreactors to see whether such substrates have any negative influences to the sludge inside. With such information, it is possible to figure out whether anaerobic conditions are met, substrate amount is optimal, or whether it is necessary to take any precautions regarding the concentrations of trace elements.

From the Figure 18 the development of dry methane content and hydrogen sulphide concentrations can be seen. At the beginning of the experiments, the sludge in both reactors was different, therefore, a difference of 10% can be seen from this graph. As well, the hydrogen sulphide concentration differed by 75 %. Afterwards, the reactors regained the comparable productivity, when they were fed with corn. It can be seen, that when the distillery waste substrate was switched to a mix of cyanobacteria-distillery waste, the methane composition of biogas has decreased by 3-4 % compared to the reactor which was always fed with distillery waste. Afterwards, the hydrogen sulphide concentration for reactor with cyanobacteria and distillery waste mix started to increase, reaching the highest value of 1180 ppm, which was constant until the end of experiments (while the highest value the device can measure was 2000 ppm). While the hydrogen sulphide concentration was observed to decrease by 60 % and methane concentration increase up to 75 % over an entire week of no feeding of reactor with distillery waste monosubstrate.

In most cases, the increase of methane content from biogas was observed after the weekend, having the highest methane content (62,15 % on day 54) development when fed

between loading rate 3 and 3,5. However, with this loading rate, the methane concentration was dropping to its lowest (51,51 % on day 43) by the end of the week.

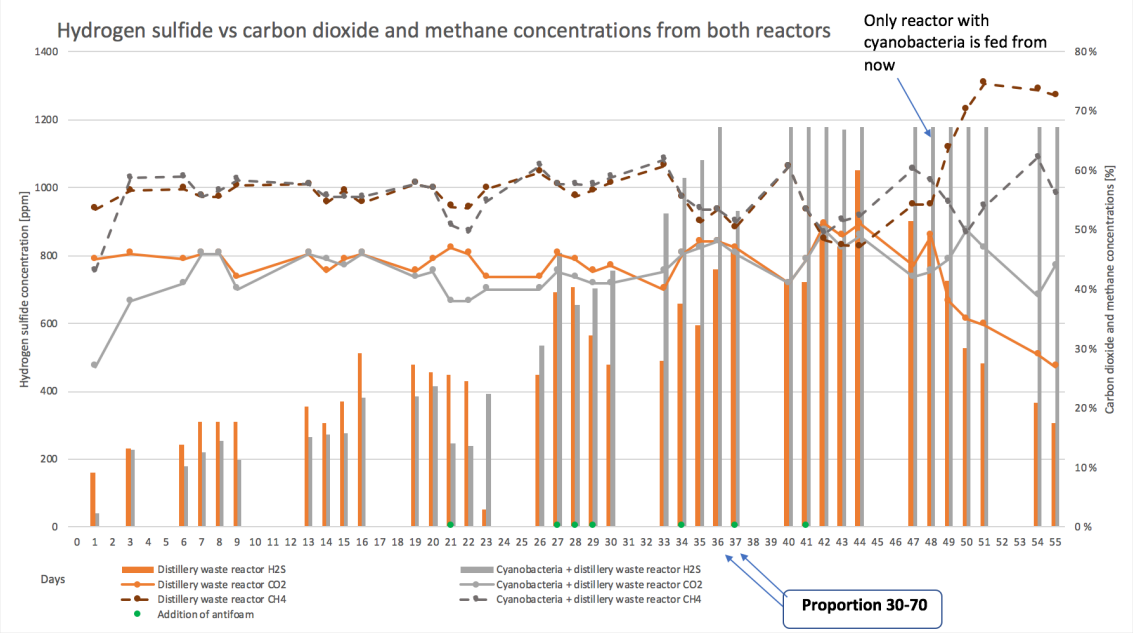


Figure 18. Hydrogen sulphide vs dry methane concentration from two continuous bioreactors (distillery waste and cyanobacteria-distillery waste reactor)

The concentration of carbon dioxide can be seen from the same figure. For simplification, the carbon dioxide concentration is proportional to methane concentration, which means that when methane level increases, the carbon dioxide concentration increases and *vice versa.* During the whole timeline of experiments, CO_2 level did not increase more than 51 %, which was observed on days 42 and 44, when the reactors were fed with loading rate 3,5. It can be seen clearly, that the levels of CO_2 were not as similar between both reactors, compared to CH_4 results. The CO_2 content was dropping by the beginning of the week of feeding, but afterwards recovering by the middle of the week.

From the Figure 19, the development of ammonium-nitrogen can be seen. The measurements started from day 9. The graph tells us that with the increase of loading rate, the ammonium-nitrogen concentration increases as well. The highest level of NH_4-N was recorded at day 50, with the concentration nearly 3000 mg/L. During the total time of experiments, reactor that was fed with distillery waste monosubstrate has showed a highest concentration of NH_4-N overall.

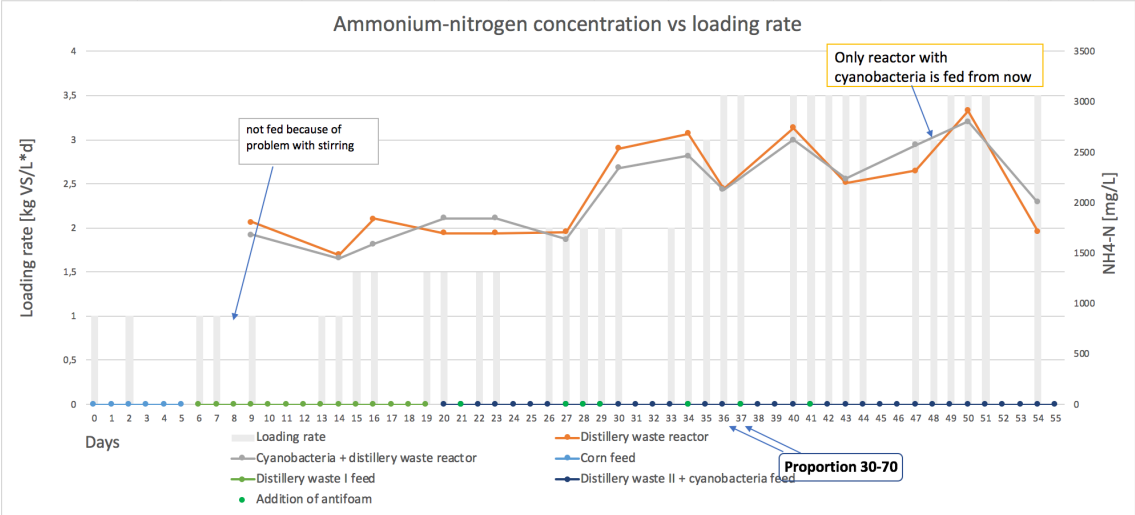


Figure 19. NH_4-N concentration against loading rate from two continuous bioreactors

From Figure 20, the development of FOS/TAC ratio can be seen. Here, during 25 days of feeding (from the start of FOS/TAC measurements), was no noticeable increase, meaning that both reactors were all this time underfed, according to the ratio description from Table 6. Afterwards, with an increased loading rate from 2 to 3,5, the sudden increase of the ratio happened, which brought both reactors to the category of overfeeding (0,5-0,6) on day 43. The loading rate was decreased to 3 for two days, where the increase of FOS/TAC ratio happens to reactor with distillery waste monosubstrate and decreases for the reactor with cyanobacteria-distillery waste mix. In order to see more detailed development, the FOS/TAC measurements were done daily, also the loading rate was returned back to 3,5, since it should not be decreased in any way. From that time, the reactor with cyanobacteria and distillery waste mix was the only one that was fed, thus the rapid decrease of FOS/TAC ratio and biogas productivity from the other reactor. The feeding with loading rate 3,5 has increased FOS/TAC ratio and kept it constant at over 0,6 for almost a week until the end of the experiments.

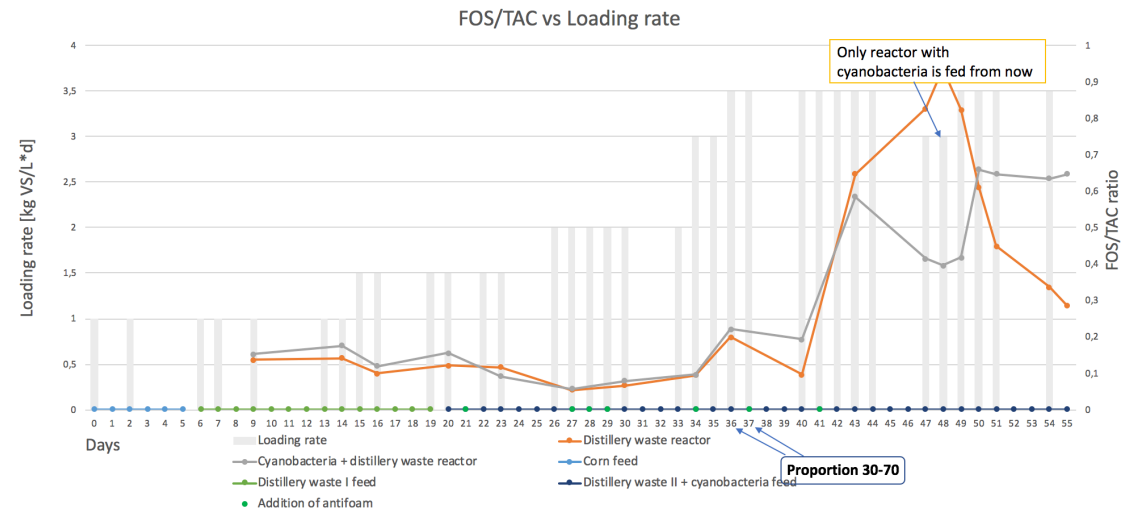


Figure 20. FOS/TAC ratio against loading rate

4.3.5 Single acid results from both reactors

Both of the fermenters (distillery waste reactor and cyanobacteria + distillery waste reactor) were analysed for the concentrations of single acids in the sludge. The acids analysed were divided to 7 groups. The representative first compounds are described from group 1 to 7: acetic acid, propanoic acid, propanedioic acid, butanoic acid, pentanoic acid, hexanoic acid. More about the concentrations and acids can be seen from Figure 21.

		Concentration (multiplied by 3) (g/l)							
	Days	14		16		20		23	
Acid groups	Acids	Reactor 5	Reactor 7	Reactor 5	Reactor 7	Reactor 5	Reactor 7	Reactor 5	Reactor 7
1	Acetic acid	0,025	0,022	0,016	0,008	0,001	0,003	0,008	0,004
2	Propanoic acid, propionic acid, carboxyethane, ethanecarboxylic acid, ethylformic acid, luprisol, luprosil	0,000	0,002	0,000	0,001	0,002	0,004	0,012	0,000
3	Propanedioic acid, dimethylmalonic acid, 2,2-propanedicarboxylic acid	0,003	0,001	0,002	0,002	0,000	0,001	0,000	0,001
4	Butanoic acid, butyric acid, n-butanoic acid, n-butyric acid, ethylacetic acid, propylformic acid, 1-butyric acid	0,013	0,009	0,015	0,008	0,018	0,005	0,047	0,027
5	3-methyl butanoic acid, isovaleric acid, beta-methylbutyric acid, isopropyl acetic acid, delphinic acid	0,010	0,007	0,013	0,007	0,020	0,007	0,049	0,030
6	Pentanoic acid	0,009	0,001	0,015	0,004	0,024	0,007	0,059	0,041
7	Hexanoic acid, n-caproic acid, n-hexanoic acid, n-hexoic acid, n-hexylic acid, butylacetic acid, caproic acid	0,002	0,002	0,005	0,003	0,011	0,003	0,008	0,016

Figure 21. Concentrations of acids from GC-MS analysis (reactor 5 – distillery waste reactor; reactor 7 – cyanobacteria with distillery waste mix reactor)

From the Figure 21, the concentrations of these acids were very low at the beginning (between 0,01-0,02 g/l). Some of these values were not able to be identified, due to a very low concentration (can be seen where concentration is 0 g/l). Although, the concentrations for both reactors were highest from the acids on 23rd day of operating bioreactors (acid groups 4, 5, 6 showed results between 0,04 and 0,06 g/l). The reason why the values were multiplied by 3, was because the samples were diluted with ratio 1:3.

Afterwards, the GC-MS device has shown some malfunctioning activity, which did not allow the further measurements of the samples for single acid concentrations.

The idea of cultivation of *Arthrospira platensis* has showed the productivity that is possible from the photobioreactor in this work. As well, it was possible to see the biogas production from the two substrates used. More about this can be seen from the next chapter.

DISCUSSION AND CONCLUSIONS

At the end of the experiments, it is possible to compare the collected results. Firstly, the idea behind feeding bioreactor with distillery waste was to identify the potential limiting effects and biogas potential, since this is not a very common substrate that is fed to bioreactors. Next, the idea behind having a cyanobacteria-distillery waste mix substrate, was to identify whether there is a possibility to fix the possible limiting effects of distillery waste used as monosubstrate. Also, the idea behind cultivating cyanobacteria was to identify the best method to produce as much biomass as possible, so that there could be a possibility to utilize the same produced biomass in the large-scale biogas production in a daily basis. Therefore, in short, such work was performed in order to optimize the closed cycle of biogas production from distillery waste substrate and utilization of produced cyanobacteria biomass.

4.4 Growth of cyanobacteria

From the collected results, it can be seen that *Arthrospira platensis* has a potential to have significant growth rate, despite of a lower concentration of the original concentration of standard medium. Especially, most significant increases were observed on the period between day 21 and day 35 from loop 3, when it was treated with return filtrate. Overall, loop 3 was identified to have 18 % higher growth rate, compared to the loop 2 (same culture with same lighting, see Table 3). Most probable reason, that the highest growth of loop 3 was due to having higher concentration of standard medium, than in the other loops with the same culture. Also, after day 50, in loop 3 was observed a decrease in growth rate, subsequently complete decline of cells (most likely due to contamination). However, the cell productivity from this loop also was in high amount; the highest value was recorded from loop 3, which was 1,55 g/l*day (108,5 grams biomass per 10 liter per week), which is much higher than the amount achieved from Bezerra et. al [6] (recorded around 0,47 g/l*day, resulting in around 32,9 g of biomass per 10 liter per week).

As for digestate loop, at the beginning, there was a slow increase in cell concentration. However, after extra addition of digestate (1,5 % v/v), by not exactly following the method in the research paper [33], the decrease in culture was noticed. Most likely, the reason no growth was the difficulty of cyanobacteria to reach the light through a very

dark medium (especially considering the issue on day 49, when lighting was not working). This might have decreased the chances of cyanobacteria to reach the amount of light needed, resulting in the decrease of culture (the light emitted to the loop was 17 W/m^2 for top lighting and 20 W/m^2 for vertical lighting, while in the research paper was around 22 W/m^2). Nevertheless, in order to solve the issue of no increase in growth rate, the addition of standard medium instead of carbonate buffer with digestate was started before the decline of cells. The addition of medium to the culture was when the loop had less contents than required (due to evaporation effect), which was usually every week, between 1-1,5 % (v/v) of the loop. Afterwards, either the cells did in fact decline, or the method of centrifugation to have blank value for measurements was not relevant anymore. This can be a good example, that it is much more difficult to shift from Erlenmeyer flasks to the mass production in photobioreactor of *Arthrospira platensis*. However, it was shown by Fait (2018) [9], that in the smaller scale the growth of cells is possible in liquid digestate medium. But still, the effect of slow growth was also noticed in the mentioned poster, where the recommended resolution of the problem would be to add digestate in different intervals, in order to avoid the scenario where cyanobacteria cannot reach light due to dark medium. Also, poor growth of the culture can be described by a very low amount of nitrogen, which was the reason to switch to standard medium in this paper. However, it did not bring any benefits to the growth of *Arthrospira Platensis* in digestate medium in this work.

4.5 Batch test biogas and methane production

The biogas production from the batch tests showed that distillery waste substrate is producing higher amount of biogas per kg VS (24 % higher), while methane volume was similar for both substrates (61 % corrected methane concentration for distillery waste substrate and 60 % for cyanobacteria-distillery waste substrate). However, since the biogas production was higher from distillery waste substrate, the methane volume per kg VS was also higher (which was around 19 % higher). Also, the hydrogen sulphide concentration was 30% higher for distillery waste substrate, but at the end of experiment, was not present anymore. These results show that distillery waste was more productive as monosubstrate, rather than working together with cyanobacteria. Most likely, in order to achieve better results, the proportion of cyanobacteria-distillery waste mix was required to be higher in respect to cyanobacteria part.

4.6 Biogas and methane production from continuous bioreactors

After the batch tests, it was possible to see whether the same biogas potential was achieved from continuous bioreactors.

The biogas and methane production have shown similar results between both substrates for almost 6 weeks of feeding. The difference was noticed from day 42 as methane concentration increase by 5-6% in reactor with cyanobacteria-distillery waste mix. This has led to a higher methane productivity from this reactor. However, it is difficult to say, whether the differences in methane concentration are because of the different substrates, or just because of the differing conditions (different sludge as described previously) in the bioreactors. As it was mentioned by Douškova et. al (2010) [34], the pseudo-steady state parameters (stabilized parameters) of distillery waste happened after 90 days of adaptation time of inoculum to the new substrate, whereas in this case, there were 49 days of feeding with distillery waste (from which 35 days feeding was with cyanobacteria-distillery waste mix). It was also told by Douškova et. al (2010) [34], that distillery waste feed matters for the methane concentration. Too high loading rate can lead to methane concentration lower than 50% (v/v). In this case, the methane concentration rarely dropped below 50 %, meaning that according to this paper the right amount of feeding was followed. However, according to FOS/TAC ratio, the reactors were always hungry, meaning that they were always underfed. From the paper, the average methane production was around 430 l/kg VS (converted from l/g COD with ratio 1:1,6 [35]), with concentration 55 %, when the total distillery waste feed was 1,61 L/day during four times of feeding per day, for 50 liters volume bioreactor. While in the case of this paper, also 287,37 l methane/kg VS was produced with average methane concentration around to 55 %, while feeding daily with 760 g fresh mass distillery waste monosubstrate in 12 liters distillery waste reactor. As for cyanobacteria-distillery waste substrate, 293,31 l methane/kg VS was produced in average, having methane concentration at 55,5 %. The produced results are around 40 % lower than from the research paper (and almost 50% lower than the results from batch tests), and both substrates seem to be very close with the methane production. Thus, it is not possible to conclude, whether cyanobacteria biomass really does improve the biogas production of distillery waste. Possibly, in order to identify, it would be necessary to increase the cyanobacteria proportion in cyanobacteria-distillery waste mix.

In addition, there was a study performed by Varol (2016) [36] where *Spirulina Platensis* was identified for biogas production if fed as monosubstrate for anaerobic digestion. Here, the results showed that in batch fermentation mode it was possible to obtain between 210 and 260 liters biogas/kg VS. While during these experiments, it was possible to have 670 and 820 liters biogas/kg VS from codigestion of cyanobacteria with distillery waste. This shows a noticeable difference, although it is not clear whether cyanobacteria monosubstrate can produce higher biogas amount than in the paper.

The production of biogas can be compared to the production of biogas from fresh maize biomass. As discussed in the paper by Dubrovskis (2010) [37] the biogas production from maize types Tango and Celido was recorded to be 476-570 l/kg VS (also under mesophilic conditions). The average methane content was between 49,6 % and 59,3 %. As from this work, when corn was fed, it was possible to produce maximum biogas around 360 l/kg VS (considering that the very short period of feeding with corn). Nevertheless, afterwards, when the reactors were fed with distillery waste and cyanobacteria-distillery waste mix, the production of biogas was highest at around 879 l/kg VS (distillery waste) and around 516 l/kg VS average biogas production over the entire feeding timeline. The biogas production of cyanobacteria-distillery waste mix reactor was highest at 709,45 l/kg VS and average biogas production was 520,56 l/kg VS during the time when fed with cyanobacteria-distillery waste mix. This could tell, that distillery waste and cyanobacteria-distillery waste mix are similar according to biogas production compared to maize. Also, as already discussed, the average methane concentration for both substrates was 55-55,5%, which is in between the range of methane concentration recorded from maize.

However, if we look at the results from batch tests, the production is twice higher, and the methane concentration is 5 % higher. This contradicts that the biogas production from distillery waste and cyanobacteria-distillery waste mix is similar to maize substrate biogas production.

4.7 Treatment of bioreactors

During the anaerobic fermentation in continuous reactors, foaming problem occurred when distillery waste substrate was changed to another distillery waste substrate. During

the foaming, the tubes that were used for biogas collection were often clogged, making it impossible to collect biogas anymore and creating pressure in the bioreactor. This can be dangerous for both microorganisms and for the surrounding, therefore foaming situation must be treated.

By researching the problem, the foaming occurrence has been explained in details by Najafpour (2015) [38]. The formation of foam happens when gas is introduced to the liquid. Additionally, any compound in the liquid that reduce surface tension may be able to influence foam formation (for example proteins can act as foam drivers). The prevention of the foam can vary, depending on the amount of proteins, fatty acids, soaps. Sometimes, foam is very easy to reduce, by using foam breaker, but it is also possible to treat foam with chemical antifoam agents. The idea is, that minimum amount must be used, since a high amount might interfere with the microbiological processes (however, for large biogas plants, high amounts might be required to be used, for example if the biogas plant is of 1000 m^3, this could mean that instead of 1 ml, 100 liters of it would be required, while ton of antifoam silicone agent costs around 4000 euros [39]). The antifoam agent that was used in this case, was based on silicone, and was very effective to reduce foaming. Afterwards, the foaming was not a big problem during the experiments and operation of bioreactors was able to be continued after first encounter of foam. There is also a thought, that the foaming problem might have occurred due to addition of substrate that was not completely thawed, which can be explained by the fact that the foam did not appear anymore after day 41 (when the substrate was warmed up before feeding).

As a second problem during biogas experiments, the hydrogen sulphide concentrations were high from both reactors. The reason for H_2S concentration was explained by Willington & Marten (1982) [1], that the distillery waste contains high suphate concentration, which during anaerobic digestion is reduced to either sulphide or hydrogen sulphide gas. Since it was not possible to filter the gas when collecting the biogas, the experiments were still continued. From reactor with cyanobacteria-distillery waste mix, the highest concentration of hydrogen sulphide was recorded (1180 ppm). This concentration is very dangerous for mammals, because while being for a short time in such an environment, it is possible to lose consciousness or death could even occur as described by [40], which is the reason that this problem must be resolved as soon as possible. By researching the issue, paper by Ma H., Chen P., Ruan R. (2001) [41] showed some methods to remove both H_2S and NH_3. Fist method, Silent Discharge Plasma (SDP) where the biogas can be

passed through the ozonisation chamber, then to multi-cell plate-to-wire (PTW) reactor, which decomposes the H_2S and NH_3 in the gas to single molecules, thus decreasing the level of odorous gas. From their experiments, 3,5 ppm concentration of H_2S was treated with successful total removal of these compounds. The efficiency of treatment varied with the increased initial concentration of dangerous compounds in the gas, and also varied with the amount of ozone injected and the humidity factor. This method would be interesting to see if the gas with H_2S concentration 1180 ppm can be treated the same way.

As for NH_4-N, the concentration was higher than usual. For this, chemical removal of ammonium-nitrogen can be done by the method described by Suschka & Poplawski [42]. With addition of superphosphate or phosphoric acid to fulfil the formula of struvite can reduce the NH_4-N levels from 2000 mg/l up to 22 mg/l.

Also, regarding single acid composition in bioreactors, it seems that there are no problems, because the concentrations of these acids are low. It did however show an increase on day 23, which was most likely the reaction to increase of loading rate. Unfortunately, it was not possible to perform any further analyses, due to some issues with GC-MS device. It was told by an expert in the field, that the single acid concentration on day 23 was not a problem. Typically, this becomes a problem when the concentration is much higher (if for example 100 times higher, resulting in 6 mg/l), and normally, a biogas plant cannot do anything about this, when the problem has occurred.

It is good to know, that the higher FOS/TAC ratio was finally achieved, since during most of the time the ratio was below 0,2. The increase was very significant, which would mean that either the reactors were fed before with lower amount of solids (although the values for liters of biogas per VS showed no such increase as FOS/TAC), or the adaptation time to the inoculum (sludge) was necessary for the used substrates.

4.8 Errors during experiments

From the experiments, some errors have occurred. For example, the sludge in the bioreactors was slightly different, which was due to some misunderstanding issues. It was required to fill up only one bioreactor with one type of sludge, but instead, it was filled up

with two different ones (same composition, but different age). However, afterwards, this did not bring noticeable differences to the results.

Also, when operating digestate loop, due to misunderstanding, additional amount of liquid digestate was added (ignoring the research paper method). This might have resulted in a problem, that the culture had no growth.

Additionally, the issue with moulding of the first batch of distillery waste has happened also due to misunderstanding issues.

Most often reason of misunderstanding was observed due to the difficulty to explain something in English, which is a common issue nowadays in the international community. However, afterwards it was possible to have better understanding, when the suggested task was discussed in the written format, rather than by verbal communication.

4.9 Outlook

From the experiments, it is possible to extrapolate the dimensions of a photobioreactor required in order to feed the produced cyanobacteria biomass to a larger scale biogas plant. With this photobioreactor it was possible to harvest 10,62 g/l in average of biomass from the best growing culture after a week, then per day it would be 1,52 g/l, while usually around 10 liters were harvested, resulting in around 15,2 g from 10 liters after a day of operation. In order to feed daily with the produced biomass, one loop should be at least 5 times larger (maybe even 7 times larger, since it might not have the same growth rate), meaning that it should be not 20 liters, but instead at least 100 liters volume. The dimensions of the loops in this photobioreactor are: 0,5 m bottom part, connected to 1,5 m vertical parts and top part 0,5 m connected to vertical parts, while 3 loops would take up around 0,5 m of space (approximately). Thus, if the same design would be followed, then the photobioreactor would occupy close to 50 m^3 of space (instead of 0,4 m^3). This would require more lighting, more energy, and more room. Therefore, if the higher dimensions are to be considered, then different design should be implemented (for example serpentine, manifold, spiral [19]), or the number of loops could be increased by 5-7 times, resulting with at least 15 loops, making the width of the photobioreactor around 2.5 meters (instead of 0,5 meters).

Let's assume, that the biogas plant volume is 1000 m^3 (roughly 100,000 times larger than the bioreactors used for this work). Thus, the feeding would be around 605 g * 100,000 = 60,5 tons per day of distillery waste 80 % composition, while for cyanobacteria it would be 18,5 tons per day as a 20 % composition with loading rate 3,5 (highest one used during this work). If the photobioreactor is planned to produce the same feeding amount, then the number of loops would be at least around 1,5 millions, resulting in a width of the same photobioreactor around 250,000 meters (250 km). For this reason, the design of this photobioreactor is not suitable for large scale feeding of biogas plant, meaning that a new design should be considered.

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APPENDICES

Appendix 1. Recorded data from the loops by operating photobioreactors with *A. platensis*

		Optical Density (750nm)					Averages				
Additional comments	Date	Loop 6	Loop 5	Loop 4	Loop 3	Loop 2	Loop 6	Loop 5	Loop 4	Loop 3	Loop 2
5 to 20 each (**AFTER DILUTION**)	28.03.2018			0,334 0,302 0,3340	0,562 0,568 0,566				0,3233	0,5651	
5 to 20 (3), 10 to 20 (4)	04.04.2018			0,627 0,63 0,591	0,998 1,022 0,957				0,6158	0,9925	
	After Dilution			0,474 0,468 0,4857	0,552 0,527 0,553				0,4759	0,5439	
10 to 20 each	11.04.2018			0,8860 0,86 0,849	0,96 0,94 0,967				0,8650	0,9558	
	After Dilution (FILTRATE)			0,542 0,517 0,548	0,636 0,617 0,662				0,5358	0,6385	
	After Dilution (FILTRATE + 1L MEDIUM SOLUTION)			0,469 0,458 0,4950	0,464 0,452 0,46				0,4740	0,4588	
10 to 20 each	18.04.2018			0,8658 0,838 0,849	1,136 1,132 1,209				0,8512	1,1591	
	After Dilution (FILTRATE)			0,558 0,526 0,549	0,288 0,275 0,284				0,5442	0,2823	
	After Dilution (FILTRATE + 1L MEDIUM SOLUTION)			0,491 0,469 0,4973	0,688 0,675 0,701				0,4859	0,6880	
2,5 to 20 liters (loops 4 and 3, 1:8 dilution). 10 liters from loop 5, 10 liters medium solution to loop 2 (1:2 dilution). 2,5 liters from both loop 3 and 4 as innoculant (5 liters) to loop 6 + 1,5% (150ml digestate), 1:2 dilution.	19.04.2018	0,417 0,4185 0,4391	0,642 0,682 0,641				0,425	0,655			
	After Dilution			0,575 0,56 0,617	0,67 0,669 0,656	0,361 0,348 0,36			0,5839	0,6649	0,3564
Loop 3, dilution 1:4 (13,5l fitrate + 1,5l medium), loops 2 and 4, dilution 1:2 (9l fitrate + 1l medium). Loop 6, finding the right blank value.	25.04.2018	0,5168 0,4804 0,4693		0,774 0,773 0,762	1,711 1,816 1,941	0,834 0,84 0,805	0,489		0,7698	1,8228	0,8264
	After Dilution			0,542 0,546 0,566	0,552 0,577 0,566	0,508 0,538 0,565			0,5514	0,5649	0,5371
Loop 3, dilution 1:4 (14l fitrate + 1,5l medium), loops 2	02.05.2018	0,5036 0,509 0,5032		0,79 0,874 0,896	1,875 1,785 1,781	0,957 0,944 0,992	0,505		0,8533	1,8137	0,9645

Aleksandr Marisev:

Appendix 2. Recorded data from the loops by operating photobioreactors with *A. platensis* (continuing)

Loop 3, dilution 1:4 (13,5l fitrate + 1,5l medium), loops 2 and 4, dilution 1:2 (9l fitrate + 1l medium). Loop 6, finding the right blank value.	25.04.2018	0,5168 0,4804 0,4693		0,774 0,773 0,762	1,711 1,816 1,941	0,834 0,84 0,805	0,489		0,7698	1,8228	0,8264
	After Dilution			0,542 0,546 0,566	0,552 0,577 0,566	0,508 0,538 0,565			0,5514	0,5649	0,5371
Loop 3, dilution 1:4 (14l fitrate + 1,5l medium), loops 2 and 4, dilution 1:2 (9,5l fitrate + 1l medium). Measuring OD of loop 6. Thawing all collected algae biomass.	02.05.2018	0,5036 0,509 0,5032		0,79 0,874 0,896	1,875 1,785 1,781	0,957 0,944 0,992	0,505		0,8533	1,8137	0,9645
	After Dilution			0,459 0,469 0,464	0,327 0,342 0,326	0,523 0,514 0,504			0,4638	0,3316	0,5136
Loop 2 1:2, 9,5L fitrate return 1 L standard solution, Loop 3 and 4, 12,6 L filtrate return and 1,2 L standard solution. Loop 6, centrifugation of sample for blank, measuring OD.	08.05.2018	0,5579 0,5772 0,5853		0,723 0,729 0,736	0,928 0,84 0,94	0,971 0,948 0,872	0,573		0,7292	0,9028	0,9304
	After Dilution			0,382 0,38 0,383	0,423 0,406 0,415	0,558 0,578 0,602			0,3815	0,4149	0,5796
Checking the OD	11.05.2018			0,436 0,432 0,451	0,548 0,533 0,525	0,689 0,708 0,684			0,4396	0,5356	0,6935
Loops 2 and 4 diluted 1:3, loop 3 diluted 1:2	16.05.2018	0,5959 0,5813 0,5967		0,748 0,714 0,749	1,358 1,35 1,336	1,094 1,058 1,066	0,591		0,7368	1,3479	1,0724
	After Dilution			0,504 0,466 0,482	0,392 0,413 0,43	0,595 0,635 0,599			0,4839	0,4116	0,6097
	18.05.2018					0,551 0,526 0,531					0,5360
	After Dilution			0,338 0,356 0,364	0,376 0,344 0,412	0,402 0,379 0,424			0,3525	0,3771	0,4016
Dilution loops 2 and 4 3:4 factor. Checking OD.	23.05.2018	0,8495 0,9049 0,9354		0,737 0,773 0,753	0,697 0,618 0,673	0,803 0,807 0,802	0,897		0,7542	0,6625	0,8037
	After Dilution										
	24.05.2018	0,6311 0,6438 0,6568		0,752 0,764 0,78	0,643 0,591 0,558	0,764 0,749 0,724	0,644		0,7651	0,5972	0,7458
	After Dilution										
Loop 2 1:2 dilution (4 liters taken as innoculate + 5 liters filtrate back, replenished with 5 liters Standard Medium). Loop 3 1:2 (8 liters innoculate added (4 from loops 2 and 4) and 10 liters Standard Medium). Loop 4 1:2 (4 liters innoculate taken out, 5 liters filtrate adde back and 5 liters Standard Medium added)	25.05.2018	0,5506 0,5209 0,5579		0,782 0,784 0,766		0,893 0,906 0,946	0,543		0,7772		0,9148
	After Dilution			0,543 0,543 0,506	0,447 0,469 0,416	0,556 0,566 0,552			0,5305	0,4441	0,5579
Loop 2 1:3, return filtrate and 700 mL SM, Loop 3 1:2, return filtrate and 800 mL SM, Loop 4 1:2, return filtrate and 500 mL SM	30.5.2018	0,5103 0,5098 0,5477		0,812 0,805 0,817	0,829 0,802 0,718	1,041 1 0,987	0,523		0,8114	0,7828	1,0093
	After Dilution			0,438 0,478 0,472	0,453 0,521 0,507	0,409 0,413 0,419			0,4627	0,4933	0,4135
Loop 2 1:3, return filtrate and 700 mL SM, Loop 3 1:2, return filtrate and 800 mL SM, Loop 4 1:2, return filtrate and 500 mL SM	06.06.2018	0,4675 0,5196 0,5173		0,853 0,848 0,875	1,113 1,031 1,097	0,908 0,984 0,951	0,501		0,8584	1,0800	0,9476
	After Dilution			0,597 0,6 0,554	0,478 0,494 0,471	0,636 0,598 0,632			0,5837	0,4811	0,6220
Loop 2 1:3, return filtrate - 400ml and 1 L SM, Loop 3 1:2, return filtrate and 1 L SM, Loop 4 1:2, return filtrate and 1 L SM	13.06.2018	-0,0878 -0,0788 -0,0528		0,815 0,818 0,86	1,133 1,037 1,044	1,185 1,166 1,164	-0,073		0,8308	1,0711	1,1718
	After Dilution			0,6 0,6 0,6	0,419 0,421 0,43	0,426 0,45 0,441			0,5999	0,4235	0,4389
Loop 2 1:3, return filtrate - (600ml disposed) and 1 L SM, Loop 3 1:2, return filtrate and 1 L SM, Loop 4 1:2, return filtrate (500ml disposed) and 1 L SM	20.06.2018	0,2411 0,2102 0,1903		0,89 0,856 0,866	0,732 0,712 0,718	1,343 1,39 1,378	0,214		0,8703	0,7211	1,3704
	After Dilution			0,517 0,489 0,487	0,573 0,567 0,583	0,436 0,421 0,428			0,4977	0,5742	0,4282
Loop 2 RESTART (5 liters from loop 3 and 4 liters from loop 4, filled up with 10 L SM (half concentration)), , Loop 3 1:2, return filtrate and 1 L SM, Loop 4 1:2, return filtrate and 1 L SM (cyanobacteria passes through filter!!!)	27.06.2018	0,3585 0,3625 0,3374		0,886 0,881 0,881	0,877 0,876 0,905	0,377 0,378 0,364	0,353		0,8827	0,8861	0,3731
	After Dilution			0,586 0,559 0,583	0,457 0,504 0,463	0,321 0,313 0,317			0,5762	0,4746	0,3170
Loop 2 RESTART (5 liters from loop 3 and 4 liters from loop 4, filled up with 10 L SM (half concentration)), , Loop 3 1:2, return filtrate and 1 L SM, Loop 4 1:2, return filtrate and 1 L SM (cyanobacteria passes through filter!!!)	27.06.2018	0,3585 0,3625 0,3374		0,886 0,881 0,881	0,877 0,876 0,905	0,377 0,378 0,364	0,353		0,8827	0,8861	0,3731
	After Dilution			0,586 0,559 0,583	0,457 0,504 0,463	0,321 0,313 0,317			0,5762	0,4746	0,3170
Loops 6, 3 and 2 only ones that left. They were taken apart this day.	02.07.2018	0,0747 0,0423 0,0467			0,402 0,418 0,414	0,56 0,576 0,572	0,055			0,4111	0,5692
	After Dilution										

Appendix 3. Calculated proportions of the fresh mass of substrates and inoculate for preparation of batch tests (including VS composition)

		Fresh mass (g)	VS (g)
Sludge	100 %	111,46	19,26
Algae	10 %	14,85	14,85
	20 %	29,70	29,70
	30 %	44,55	44,55
	100 %	148,51	9,46
Distillery II	70 %	86,78	86,78
	80 %	99,17	99,17
	90 %	111,57	111,57
	100 %	123,97	9,63

			Fresh mass (g)	VS	Sum	Sum VS	Sludge composition	VS sludge	VS Substrate/VS Innoculate	
Algae + distillery	10-90%	Algae	14,85	0,95	126,42	9,62	1373,58	19,23	**0,50**	
		Distillery	111,57	8,67						
	20-80%	Algae	29,70	1,89	128,88	9,60	1371,12	19,20	**0,50**	Fed with this one
		Distillery	99,17	7,71						
	30-70%	Algae	44,55	2,84	131,33	9,58	1368,67	19,16	**0,50**	
		Distillery	86,78	6,74						
Distillery	100 %	Distillery	123,97	9,63	123,97	9,63	1376,03	19,26	**0,50**	

Appendix 4. Calculated TS and VS values for substrates

							Triplicates		Average	
	Crucibles	Crucible mass (g)	moist mass (g)	mass (g)	after 105 degrees (g)	after 550 degrees (g)	TS	VS	TS	VS
Distillery	Sample 1	79,42	179,42	100	90,66	79,9	11,24 %	10,76 %		
	Sample 2	91,82	191,82	100	103,11	92,31	11,29 %	10,80 %	11,25 %	10,77 %
	Sample 3	73,5	173,5	100	84,73	73,99	11,23 %	10,74 %		
Algae	Sample 1	76,64	126,64	50	80,65	77,4	8,02 %	6,50 %		
	Sample 2	82,09	132,09	50	86,03	82,85	7,88 %	6,36 %	7,87 %	6,37 %
	Sample 3	86,45	136,45	50	90,31	87,18	7,72 %	6,26 %		
	Crucibles	Crucible mass (g)	moist mass (g)	mass (g)	after 105 degrees (g)	after 550 degrees (g)	TS	VS	TS	VS
Distillery II	Sample 1	90,41	190,41	100	100,43	91,08	10,02 %	9,35 %		
	Sample 2	81,16	181,16	100	90,21	81,8	9,05 %	8,41 %	8,42 %	7,77 %
	Sample 3	84,23	184,23	100	90,42	84,88	6,19 %	5,54 %		
	Crucibles	Crucible mass (g)	moist mass (g)	mass (g)	after 105 degrees (g)	after 550 degrees (g)	TS	VS	TS	VS
Sludge	Sample 1	81,03	181,03	100	83,53	82,11	2,50 %	1,42 %		
	Sample 2	84,1	184,1	100	86,6	85,19	2,50 %	1,41 %	2,48 %	1,40 %
	Sample 3	90,3	190,3	100	92,75	91,37	2,45 %	1,38 %		

Appendix 5. Calculated feeding of the fresh mass of substrates according to specific loading rates (including VS composition)

	Proportions	daily feed (g)	Loading rate 1	g VS LR1	Loading rate 1,5	g VS LR1,5	Loading rate 2	g VS LR2	Loading rate 2,5	g VS LR2,5	Loading rate 3	g VS LR3	loading rate 3,5	g VS LR3,5	Loading rate 4	g VS LR4
Distillery	90 %	100,31	140,43	15,12	210,65	22,68	280,87	30,24	351,08	37,80	421,30	45,36	491,52	52,92	561,73	60,48
	80 %	89,16	124,83	13,44	187,24	20,16	249,66	26,88	312,07	33,60	374,49	40,32	436,90	47,04	499,32	53,76
	100 %	111,46	156,04	16,80	234,06	25,20	312,07	33,60	390,09	42,00	468,11	50,40	546,13	58,80	624,15	67,20
Algae	10 %	18,83	26,36	1,68	39,54	2,52	52,72	3,36	65,90	4,20	79,08	5,04	92,26	5,88	105,44	6,72
	20 %	37,66	52,72	3,36	79,08	5,04	105,44	6,72	131,80	8,40	158,16	10,08	184,52	11,76	210,88	13,44
	30 %	56,49	79,08	5,04	118,62	7,56	158,16	10,08	197,70	12,60	237,24	15,12	276,78	17,64	316,32	20,16
	40 %	75,31	105,44	6,72	158,16	10,08	210,88	13,44	263,60	16,80	316,32	20,16	369,04	23,52	421,76	26,88
Distillery II	90 %	139,06	194,68	15,12	292,02	22,68	389,36	30,24	486,70	37,80	584,03	45,36	681,37	52,92	778,71	60,48
	80 %	123,61	173,05	13,44	259,57	20,16	346,09	26,88	432,62	33,60	519,14	40,32	605,67	47,04	692,19	53,76
	70 %	108,15	151,42	11,76	227,12	17,64	302,83	23,52	378,54	29,40	454,25	35,28	529,96	41,16	605,67	47,04
	60 %	92,70	129,79	10,08	194,68	15,12	259,57	20,16	324,46	25,20	389,36	30,24	454,25	35,28	519,14	40,32
	100 %	154,51	216,31	16,80	324,46	25,20	432,62	33,60	540,77	42,00	648,93	50,40	757,08	58,80	865,24	67,20

		g VS LR1,5	FRESH 1,5	Total
Algae	20 %	5,04	338,65	25,2
Distillery II	80 %	20,16		
Algae	30 %			
Distillery II	70 %			

		g VS LR2	FRESH 2	Total
		6,72	451,53	33,6
		26,88		40,32

		g VS LR3	FRESH 3	Total
		10,08	677,30	50,4
		40,32		47,04
				17,64
				41,16

		g VS LR3,5	FRESH 3,5	Total
		11,76	790,18	58,8
		47,04		
		17,64	806,74	58,8
		41,16		

		g VS LR4	FRESH 4	Total
		13,44	903,07	67,2
		53,76		

Appendix 6. Table with collected biogas results from batch tests

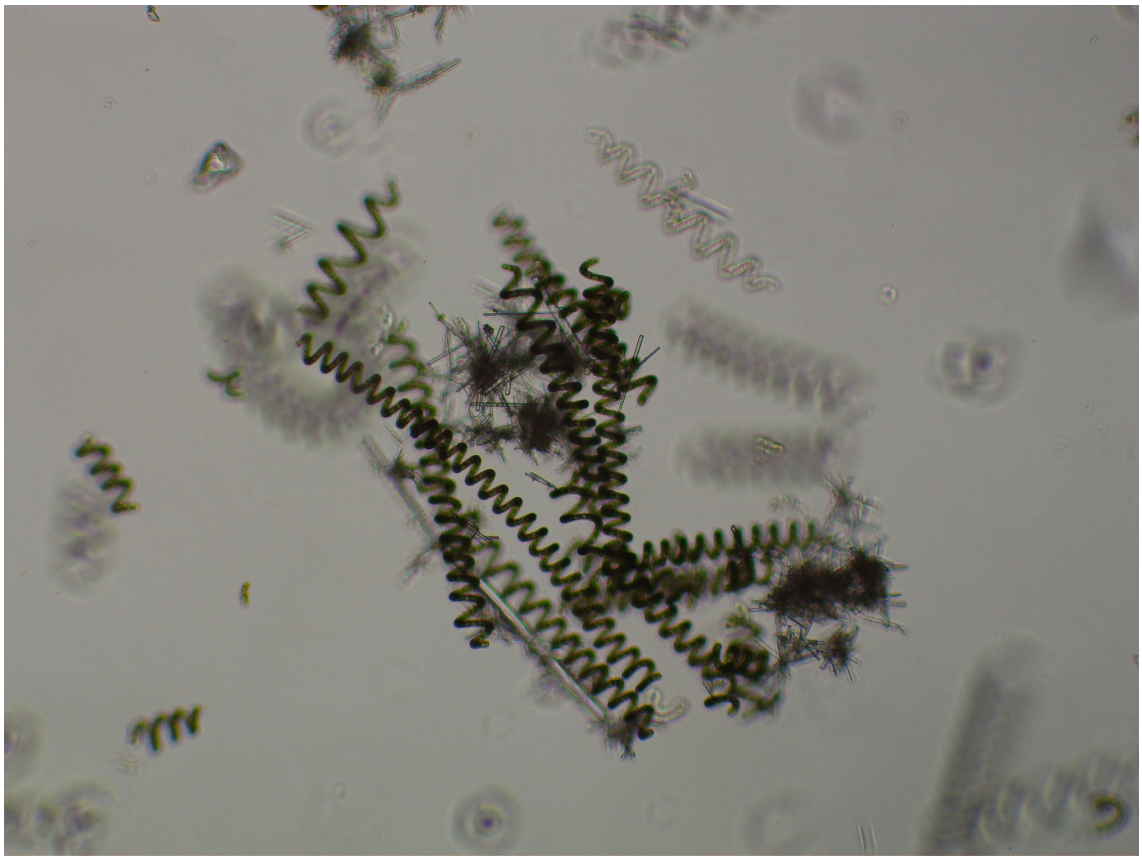
Comments	Date	Time	Temp (°C)	Pressure mbar	Pw mBar	DW I CH4 %	CH4 % dry	CH4 % corr	CO2 %	H2S ppm	Start liters	End liters	Difference liters	**Norm gas volume**	V methane	DW II CH4	CH4 % dry	CH4 % corr	CO2	H2S	Start	End	Differe	**Norm gas volume**	V methan
START	07/06/2018																								
	08/06/2018	10.19	25	1002,6	31,75	36	37,18	42,24	43	40	170,65	174,48	3,83	**3,67**	1,55	36	37,18	41,59	44	26	174,48	178,88	4,4	**4,22**	1,75
	11/06/2018	10.20	23,4	1002,3	28,84	62	63,84	70,95	37	26	324,55	326,5	1,95	**1,87**	1,33	61	62,81	70,65	36	18	326,5	328,2	1,7	**1,63**	1,15
	13/06/2018	10.42	22,3	1001,6	26,96	62	63,72	63,69	39	34	397	400,12	3,12	**3,00**	1,91	61	62,69	62,67	39	36	400,12	403,12	3	**2,89**	1,81
	20/06/2018	9.10	23,2	1011,1	28,50	61	62,77	62,58	40	18	112,4	115	2,6	**2,52**	1,58	61	62,77	62,79	41	20	115	117,4	2,4	**2,33**	1,46
	12/07/2018	11.47	25,1	1004,3	31,94	59	60,94	60,36	36	0	90,65	92,29	1,64	**1,57**	0,95	58	59,90	58,92	46	0	92,29	93,8	1,51	**1,45**	0,85

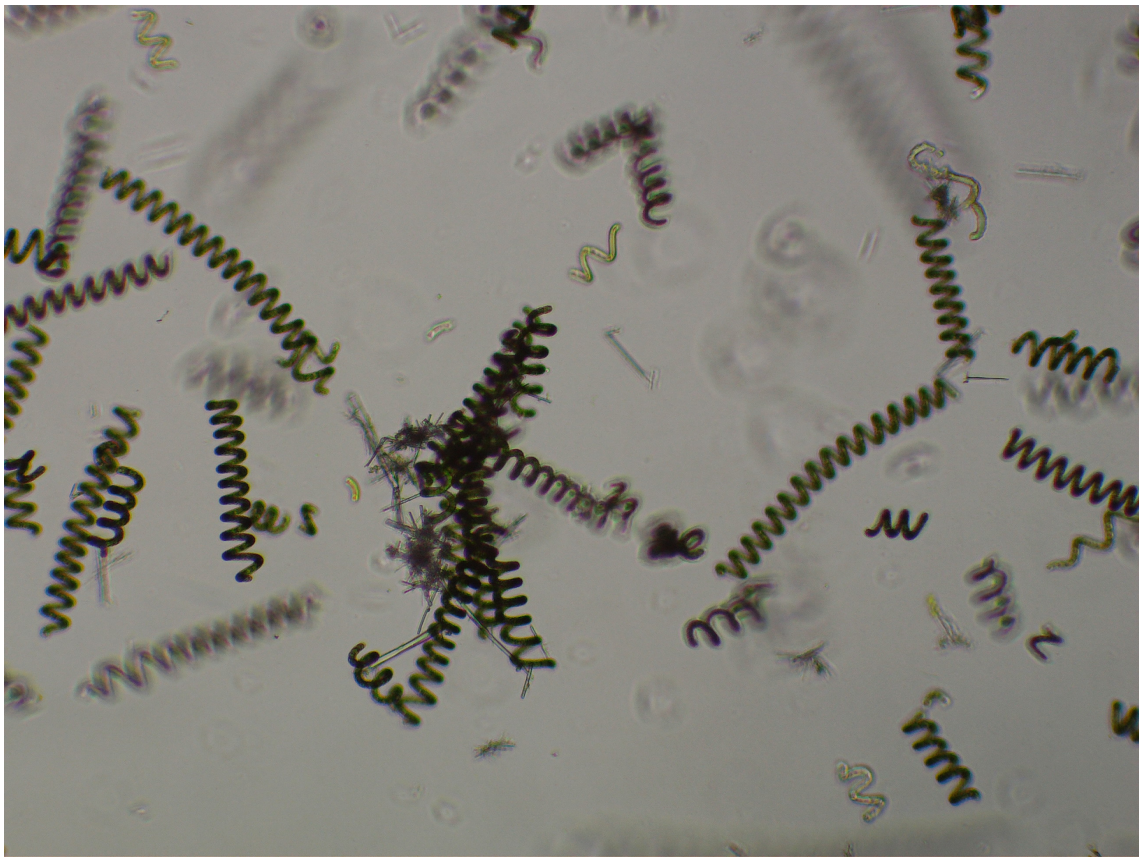
Negative Contol 1										**Negative Control 2**									
CH4	CH4 % dry	CH4 % corr	CO2	H2S	Start	End	Difference liters	**Norm gas volume**	V methane	CH4	CH4 % dry	CH4 % corr	CO2	H2S	Start	End	Difference liters	**Norm gas volume**	V methane
49	50,61	61,33	32	0	85,59	88,05	2,46	**2,36**	1,45	47	48,54	58,27	31	2	88,05	90,65	2,6	**2,50**	1,45

DW + CB I										**DW + CB II**									
CH4	CH4 % dry	CH4 % corr	CO2	H2S	Start	End	Difference liters	**Norm gas volume**	V methane	CH4	CH4 % dry	CH4 % corr	CO2	H2S	Start	End	Difference liters	**Norm gas volume**	V methane
38	39,24	44,70	43	56	182,7	186,4	3,75	**3,59**	1,61	37	38,21	43,64	42	52	178,9	182,7	3,77	**3,61**	1,58
65	66,93	76,22	34	32	328,2	329,8	1,55	**1,49**	1,14	64	65,90	73,28	35	36	329,8	331,7	1,95	**1,87**	1,37
63	64,74	64,36	39	42	403,1	406,1	3	**2,89**	1,86	62	63,72	63,21	37	50	406,1	408,4	2,25	**2,16**	1,37
61	62,77	62,20	41	28	117,4	119,2	1,8	**1,75**	1,09	60	61,74	61,27	39	16	119,2	121,4	2,15	**2,09**	1,28
61	63,00	63,25	41	28	93,8	94,3	0,5	**0,48**	0,30	59	60,94	60,59	36	0	94,3	95,49	1,19	**1,14**	0,69

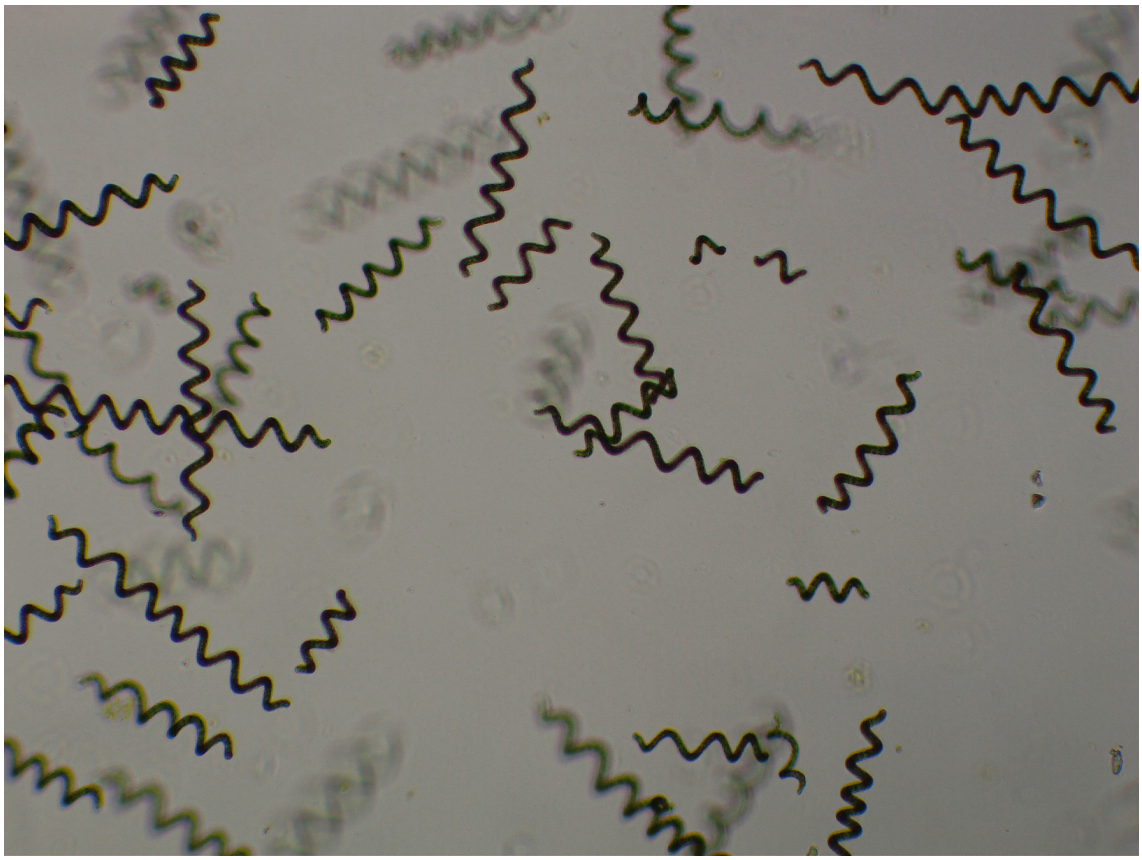
Appendix 7. Table with collected biogas results from continuous reactors

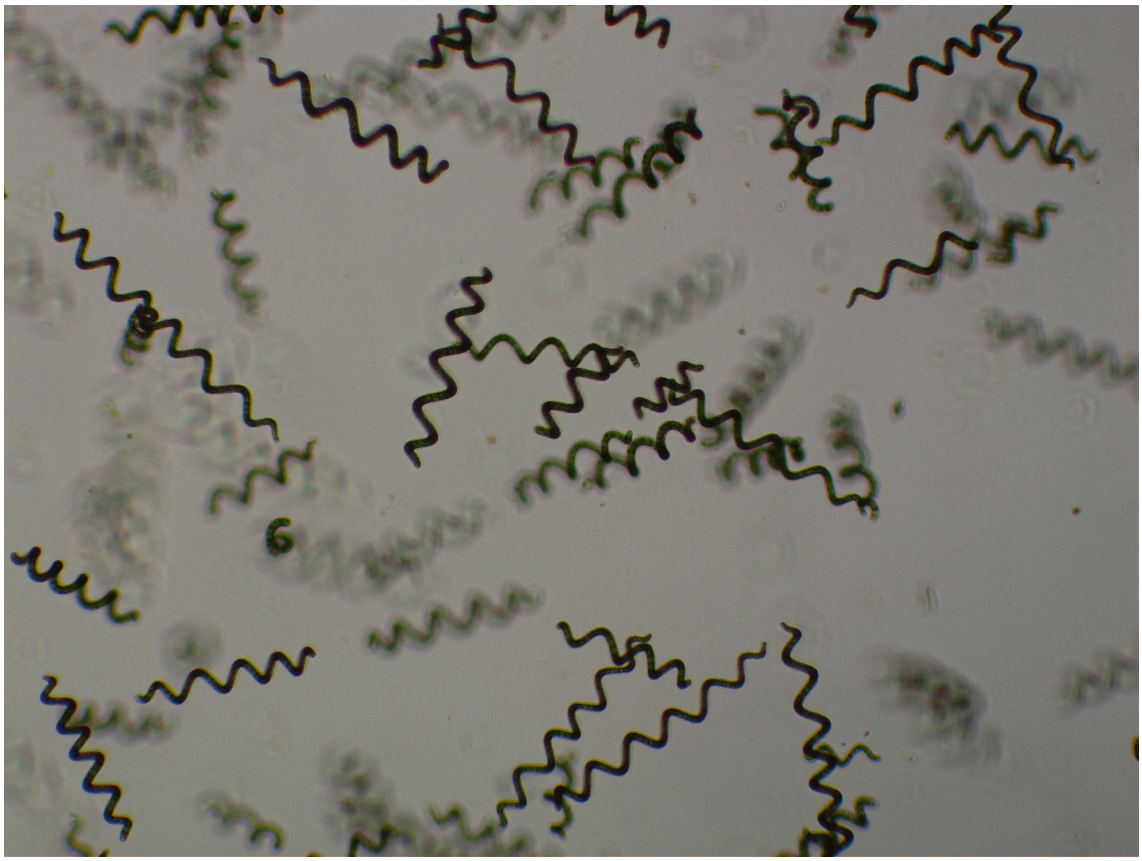
Appendix 8. Two pictures of loop 2 (*Arthrospira platensis)* 400x zoom with microscope



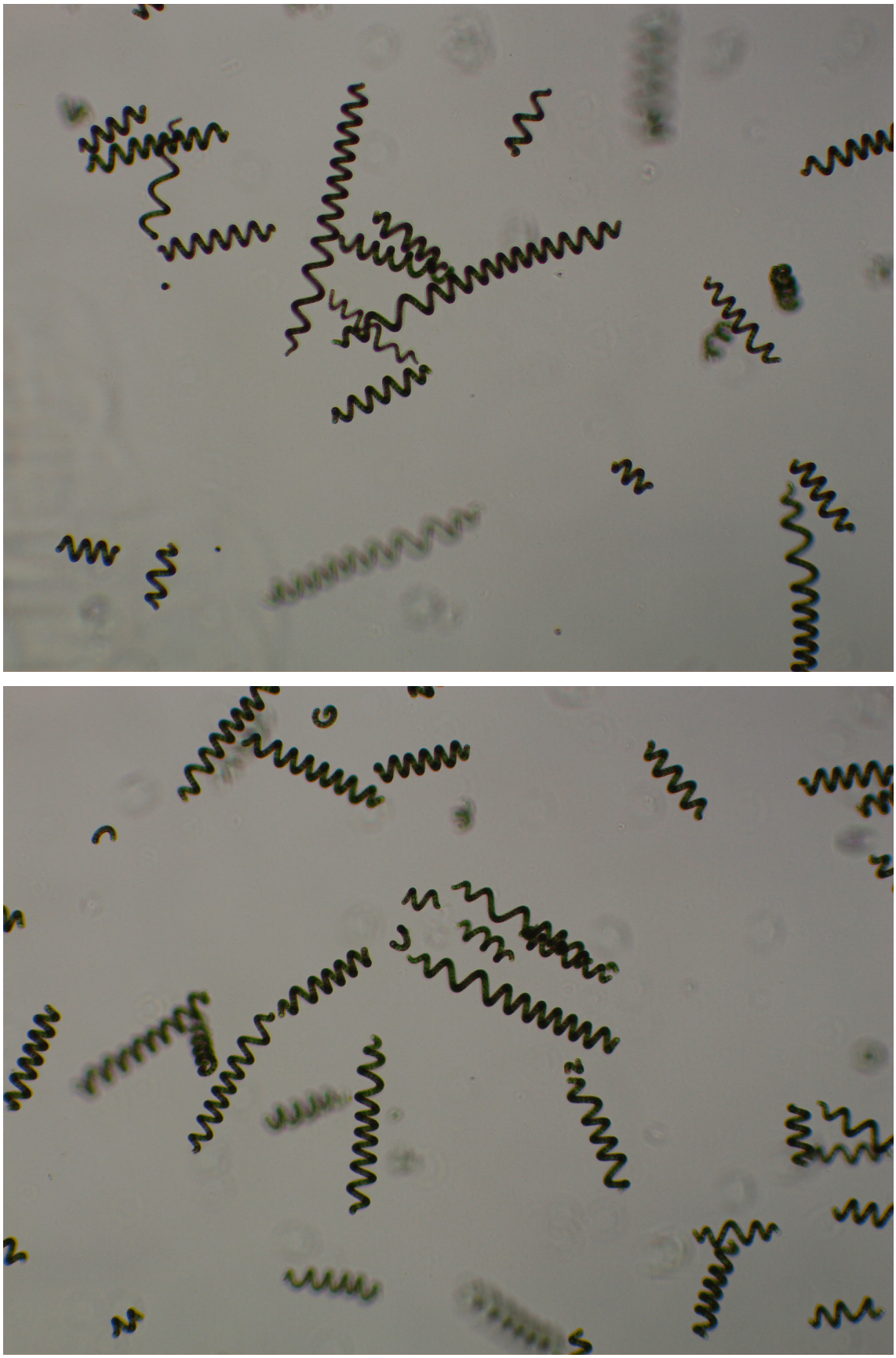


Appendix 9. Two pictures of loop 3 (*Arthrospira platensis)* 400x zoom with microscope





Appendix 10. Two pictures of loop 4 (*Arthrospira platensis)* 400x zoom with microscope



Appendix 11. Two pictures of loop 6 (*Arthrospira platensis* with liquid digestate*)* 400x zoom with microscope